DOCTORAL THESIS

Measuring Gene Expression in Endurance Athletes as a Novel Technique for Determining training Response to Sprint Interval Training (SIT)

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Measuring Gene Expression in Endurance Athletes as a Novel Technique for Determining training Response to Sprint Interval Training (SIT)

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Abstract

The fundamental aim of an endurance athlete is to improve their ability to physically perform at a high intensity for a prolonged period of time. Furthermore, this high performance area of sport can be metabolically demanding, requiring high rates of oxidative (aerobic) and non-oxidative (anaerobic) metabolism with precise regulation and control. To meet these requirements, monitoring of training is an essential component of a successful training plan, as manipulation of training volume, frequency, intensity and recovery is vital for optimal results. Due to the long-distance nature of endurance events, high-volume and low-intensity training has traditionally been the main focus for endurance athletes. However, this high-volume load can often lead to overtraining and/or a performance plateau. More recently, a shift in training volume and intensity involving low volume but high intensity sessions (termed high-intensity interval training; HIT), has become more commonly used by endurance athletes. While endurance athletes have long appreciated the role of HIT as part of a comprehensive training program, the recent surge in HIT popularity is mainly due to the efficiency of the training and the recent evidence that it causes gene expression and phenotypic performance changes that resemble those of endurance training. Studies have suggested that in young healthy persons of average fitness, intense interval training is therefore a time-efficient strategy with results comparable to traditional endurance training. However, despite recent research advancements, the fundamental question remains regarding the minimum volume of exercise necessary to improve performance.

The aim of the proposed study was to investigate two different regimes of high intensity exercise (low-volume/high-frequency and high-volume/low-frequency) in 26 well-trained endurance cyclists, measure their effect on both physiological changes and gene expression changes, and determine whether the changes caused by HIT are due to total work or are regime-dependent. The training intervention consisted of nine bouts of 30-second sprints per week for two weeks at different volumes per group: one low-volume/high-frequency group with 3 repetitions 3 times per week (9 subjects), one high-volume/low-frequency group with 9 repetitions once per week (9 subjects), and one control group without training intervention (8 subjects). The physiological measures of interest related to the metabolic

changes from the endurance capacity test, VO_2max test, and Wingate test, which were measured on all subjects at time-points before (baseline) and after the intervention period. To achieve a full understanding of the biochemical adaptation, regulation and markers associated with HIT, an examination of the gene expression changes *in vivo* was performed by taking blood samples from participants before training (baseline), immediately after the final training session (acute response), and 72 hours after the final training session (delayed response), extracting RNA from white blood cells, and undertaking a genome-wide microarray analysis to identify genes differentially expressed after high-intensity exercise. The aim was also to investigate the changes in expression levels in response to varying levels of exercise (frequency of weekly sessions and number of bouts per session), and which of these training regimes would achieve a higher performance outcome.

Some significant differences were found in the physiological traits measured between training intervention groups and the control group, as well as between the groups themselves. While there were no significant changes in ventilation threshold (VT1) between the two training groups themselves, a significant difference was found between both of the training groups and the control group post-training. Furthermore, a significant increase from baseline ($p = 0.006$) was found in the post-training intervention endurance capacity test (ECT) for the high-volume/low-frequency group, while no significant change was seen in the low-volume/high-frequency group or the control group. These findings suggest that the high-volume/low-frequency regime may be more effective at improving endurance performance in relation to the endurance capacity test.

In relation to gene expression changes, data was only available for 6 subjects in the low-volume/high-frequency group and 4 subjects in the high-volume/low-frequency group. Microarrays were performed for these subjects at three time-points (baseline, acute post-training and delayed post-training); however, after normalisation, quality control and statistical analysis of participant data, no significant changes in gene expression were found between either of the two post-training time-points compared to baseline, or between different training regime groups.

Overall, however, this study allowed researchers to obtain an increased understanding of the physiological and gene expression adaptations that can result from high intensity training and determined that training regime influences performance outcome. By implementing the minimum training regime necessary to obtain performance improvements it may be possible to optimise athlete response to training whilst avoid overtraining and illness. Additionally, no alteration in gene expression was detected after the training intervention, possibly due to that the effect size investigated being smaller than anticipated. Further research in this area may provide sport coaches, exercise physiologists, sport scientists, and athletes another tool to optimise training prescription for athletes as well as evaluating and monitoring an individual's biological response to exercise.

Declaration of Originality

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy. This thesis represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this University or any other institution, except where due acknowledgement is made.

Signed:

Siri Lauluten Szlezak

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"Far better it is to dare mighty things, to win glorious triumphs, even though checkered by failure, than to take rank with those poor spirits who neither enjoy much nor suffer much, because they live in the gray twilight that knows not victory nor defeat."

Theodore Roosevelt

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List of Abbreviations

2,3-DPG	2, 3-diphosphoglycerate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
AT	Anaerobic threshold
ATP	Adenosine triphosphate
ATP-PC	Phosphagen system
a-vO_2	Arterio-venous oxygen difference
BTPS	Body temperature and pressure, saturated
Ca^{2+}	Calcium (ionised)
CaO_2	Arterial oxygen content
CCD	Charge-coupled device
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CoA	*Coenzyme A*
COPD	Chronic obstructive pulmonary disease
CDT	Constant duration test
CPT	Constant power test
CWT	Constant-work test
CP	Creatine phosphate

CPT1B	Carnitine palmitoyltransferase 1B
CRAT	Carnitine O-Acetyltransferase
CvO_2	Mixed venous O_2 content
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ECG	Electrocardiogram
ECT	Endurance capacity test
EMG	Electromyography
ERK1/2	Extracellular-signal-regulated kinases
FAD	Flavin adenine dinucleotide (oxidised)
$FADH_2$	Flavin adenine dinucleotide (reduced)
FC	Fold change
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRP58	58 kDa glucose-regulated protein
GTP	Guanosine triphosphate
H_2O	Dihydrogen monoxide/water
Hb	Haemoglobin
HIT	High intensity interval training
HK	Hexokinase
HR	Heart rate
HRV	Heart rate variability
JNK	c-jun NH_2-terminal kinase

log_2	Logarithm to the base 2
LSD	Long slow distance training
LT	Lactate threshold
MAPK	Mitogen-activated protein kinases
MAPKAPK1	Mitogen activated protein kinase activated protein kinase 1
mRNA	Messenger RNA
MSK	Mitogen- and stress-activated protein kinase
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
O_2	Oxygen (molecular)
OCTN2	Carnitine/organic cation transporter
PaO_2	Partial pressure of O_2 in arterial blood
PBMC	Peripheral blood mononuclear cell
PCO_2	Partial pressure of carbon dioxide
PCR	Polymerase chain reaction
PFK	Phosphofructokinase
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
P_i	Inorganic phosphate
RCT	Respiratory compensation threshold
RER	Respiratory exchange ratio
R-I	Ratio-intensity
RNA	Ribonucleic acid
RPE	Rating of perceived exertion

RQ	Respiratory quotient
RT-PCR	Reverse transcription polymerase chain reaction
S6K	SK Kinase
SAGE	Serial analysis of gene expression
SaO_2	Saturation level of oxygen in haemoglobin
SD	Standard deviation
SEM	Standard error of the mean
SIT	Sprint interval training
TCA cycle	Tricarboxylic acid cycle
TT	Time trial
VCO_2	Volume of carbon dioxide
V_E	Minute Ventilation
VEGF	Vascular endothelial growth factor
VO_2max	Maximum oxygen consumption
VST	Variance-stabilizing transformation
VT1	Ventilation threshold 1
VT2	Ventilation threshold 2
WAnT	Wingate anaerobic test
WBC	White blood cells

Publications Arising from Thesis

S. Lauluten Szlezak, G. Gass, A. M. Szlezak, B. Gray. “Mouthpiece or facemask? Evaluation of optimal spirometry apparatus during continuous high intensity exercise.” *Isokinetics and Exercise Science* (2015). [Manuscript in preparation.]

S. Lauluten Szlezak, A. M. Szlezak, J. Keane, B. Gray. “Improved athletic performance in trained endurance cyclists after high-intensity sprint interval training (SIT).” *Journal of Science and Medicine in Sport* (2015). [Manuscript in preparation.]

Contribution to jointly published work

S. Lauluten Szlezak, G. Gass, A. M. Szlezak, B. Gray. "Mouthpiece or facemask? Evaluation of optimal spirometry apparatus during continuous high intensity exercise." *Isokinetics and Exercise Science* (2015). [Manuscript in preparation.]

The idea of this project was generated by the candidate and B. Gray. The data collection procedures were undertaken by the candidate, G. Gass and A. M. Szlezak. The analysis of the data was undertaken by the candidate, G, Gass and A. M. Szlezak. The production of the manuscripts is undertaken by the candidate, B. Gray and A. M. Szlezak

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The idea of this project was generated by the candidate and B. Gray. The data collection procedures were undertaken by the candidate, J. Keane and A. M. Szlezak. The analysis of the data was undertaken by the candidate, the candidate and A. M. Szlezak. The production of the manuscripts is undertaken by the candidate, B. Gray and A. M. Szlezak and J. Keane

Chapter 1 - Introduction

"Patience and perseverance have magical effect before which difficulties disappear and obstacles vanish" - John Quincy Adams.

The fundamental aim of endurance athletes is to improve their ability to physically perform at high exercise intensity over a prolonged pre-determined running distance. A general issue common to endurance athletes, coaches and researchers arises as to how effectively one can monitor, by optimal means, the athlete's performance progress in response to their training. This remains a fundamental question to achieve the desired exercise-based performance outcomes. Monitoring of training is an essential component and is accentuated to become a successful training plan by determining diverse parameters such as the manipulation of exercise volume, frequency, intensity and recovery, all of which are vital to reach optimal results.

The primary choice of exercise stimulus that endurance athletes such as Marathon runners or Tour de France cyclists opt for typically involves long slow distance (LSD) training. This type of training relies on oxidative phosphorylation as the main metabolic source, (Verde et al., 1992; Powers & Howley, 2007). LSD consists of a continuous training style that is performed at a constant pace of low to moderate intensity and over an extended distance and duration (Powers & Howley, 2007). This type of training has been shown to generate major adaptations in biochemical aerobic metabolism by means of enhanced oxygen consumption and an increase in oxidative enzymatic activity and numbers (Coyle, 1999; Laursen & Rhodes, 2001; Joyner & Coyle, 2008), resulting in enhanced oxygen consumption and greater efficiency of oxygen use. Based on the literature, elite cyclists undertaking a training regime of this kind typically cover distances of approximately 20,000 – 35,000 kilometres per year (Lucia et al., 2000; Faria et al., 2005a). The Tour de France contestant, Thor Hushovd, who cycled 1885 km in one week, illustrates this level of dedication during training. Precise recording data of Hushovd's training time was undertaken and consisted of 31 hours and 52 minutes, for which 30 hours and 30 minutes of that total time was performed at a slow pace (Hansen, 2011).

However, this high exercise volume often leads to overtraining which can lead to the athlete reaching a performance plateau (Faria et al., 2005b). Additionally, a regime involving this scale of distances can be associated with overuse injuries and disabilities (Faria et al., 2005b). Monitoring of training becomes essential to lead and promote an optimal safe and enhanced performance. Successful endurance athletes possess high aerobic capacity (~74 $mL^{-1} \cdot kg^{-1} min^{-1}$ and as a consequence consume large volumes of oxygen, a direct physiological relation enabling them to maintain high power outputs (Lucia et al., 1999; Padilla et al., 1999; Faria et al., 2005a). In training sessions, VO_2max/peak measurements are recommended for the purpose of optimally evaluating and monitoring endurance athletes (Coyle, 1999; Howley et al., 1995; Faria et al., 2005a). However, additional early detection tools are lacking for monitoring the training of these athletes or exercise enthusiasts.

Maximal oxygen consumption (VO_2max) is one of the most important measurements made in the assessment of human endurance capacity. VO_2max provides an index of cardiopulmonary fitness and aerobic capacity that are related to endurance performance (Bergh et al., 2000). A VO_2max test is therefore used to characterise subjects in research by reflecting the expressed or prescribed exercise intensity or training (using %VO_2max) on individuals of the study. It will also monitor the effectiveness of such endurance training programs that the participants are subjected to. While VO_2max is assessed as the highest level of oxygen that can be consumed, the endurance capacity test (ECT) is an additional useful measure to adopt when assessing endurance performance. The ECT utilises a constant speed and/or power output until volitional fatigue is reached. This test has been reported in the scientific literature as a reliable tool in monitoring exercise training and importantly, is a valid test for reflecting aerobic endurance performance (Bosquet et al., 2002). In contrast to both VO_2max and ECT, two highly related tools used in endurance performance, an additional test aimed at measuring peak anaerobic power and anaerobic capacity can be undertaken. Such a technique is known as the 30-second Wingate test, which is widely used in cyclists and is performed on a cycle ergometer. This test is used to validate this type of exercise and is highly relevant to cyclists.

Measures that are traditionally used in monitoring performance changes of endurance-trained athletes include VO_2max, blood lactate and cardiovascular-based readings such as heart rate. However, at the time of the performance measure a caveat with current monitoring techniques exists as functional adaptations have already taken place. These physiological, exercise-based adaptations are currently not appropriately defined as to their processes and real performance efficacy status following exercise training. Performance might have reached an adaptation plateau or could be subject to overreaching. Over-reaching is recognised as a problem in sport and exercise training that challenges both athletes and coaches and is currently associated with a poor understanding of the processes occurring in exercise adaptation. A better scientific knowledge of such processes is necessary and will enable the detection of performance decrements at early training stages. Further research is necessary to monitor performance progress and efficacy.

Elite athletes such as professional road-cyclists cover great distances daily when performing their training sessions, which can range from 5 to 300 kilometres per day. In great competitive sport events such as the Tour de France, a multi-stage event of high demand, athletes can cover up to 3899 to 4352 kilometres for the total race (Lucia et al., 2003a; Lucia et al., 2003b; Faria et al., 2005a). In these races, surges and sprints to the finish line are events that captivate the whole world of cycling enthusiasts. This particular type of exercise demonstrates the diversity in metabolic demands performed by these athletes. Effectively, it is well documented that these athletes use, at different times during the event, the adenosine triphosphate-phosphocreatine (ATP-PC) energy system and the common glycolytic and oxidative phosphorylation pathways (Lucia et al., 2003b; Faria et al., 2005b). ATP is a nucleoside triphosphate, the cellular energy resource that, once broken down, releases the energy responsible for muscular contraction. The processes required to regenerate this essential molecule are proficient in athletes and rely on a regulated multi-level metabolic pathway.

However, ATP-phosphocreatine (ATP-PC) lasts for approximately 0-3 seconds (Brooks et al., 2004) and is consumed during events such as a “dead-lift”. Glycolysis and glycogenolysis, which involve the breakdown of glucose and glycogen, respectively (Brooks et al., 2004) sustain the provision of ATP for muscular activity up to approximately 50 seconds (Brooks et

al., 2004). Glycolysis is the anaerobic pathway mainly in use during events like 200 m sprints, while exercise which lasts longer than approximately two minutes cannot be sustained without the contribution of ATP resulting from oxidative phosphorylation (Brooks et al., 2004). This aerobic based process involving mitochondria is used during events such as Marathon runs. Therefore, of the three energy systems, two systems (ATP-PC and glycolysis) do not require oxygen for their operation, while oxidative phosphorylation is the unique aerobic energy system. This latter system is dependent on the presence of oxygen as this molecule is the final acceptor of electrons which are conducted along the electron transport chain within the mitochondria (Brooks et al., 2004). In order to target these metabolic systems, training variables must be studied with a means of gathering data on exercise frequency, intensity, volume, duration and modes of exercise. This phenotypic data is indicative of metabolic processes but interestingly, is also associated with cellular responses and cellular adaptations to training conditions. Cellular specific gene expression patterns can be assessed using such training factors and once captured may reflect well the interrelated metabolic changes observed in athletes during exercise. These patterns are also dynamic and can be studied when manipulating these exercise based training demands and factors (Bickel et al., 2005).

VO_2max/peak is the maximum capacity of an individual's body to transport and use oxygen during incremental exercise. During a VO_2max/peak test, there are three points that are specifically used for performance measurement purposes. The first sudden change in gradient (see Figure 1) reflects the anaerobic threshold (AT) or ventilation threshold 1 (VT1), the second sudden change in gradient reflects the respiratory compensation threshold (RCT) or ventilation threshold 2 (VT2) (see Figure 1), and the third point is VO_2max/peak (see Figure 2). The AT and RCT are characterised by disproportionate increases in expired minute ventilation (V_E) in relation to VO_2 consumption and carbon dioxide (CO_2) production (VCO_2) during incremental exercise (Cross et al., 2012).

Figure 1. Anaerobic threshold (AT) or VT1 and respiratory compensation threshold (RCT) or VT2

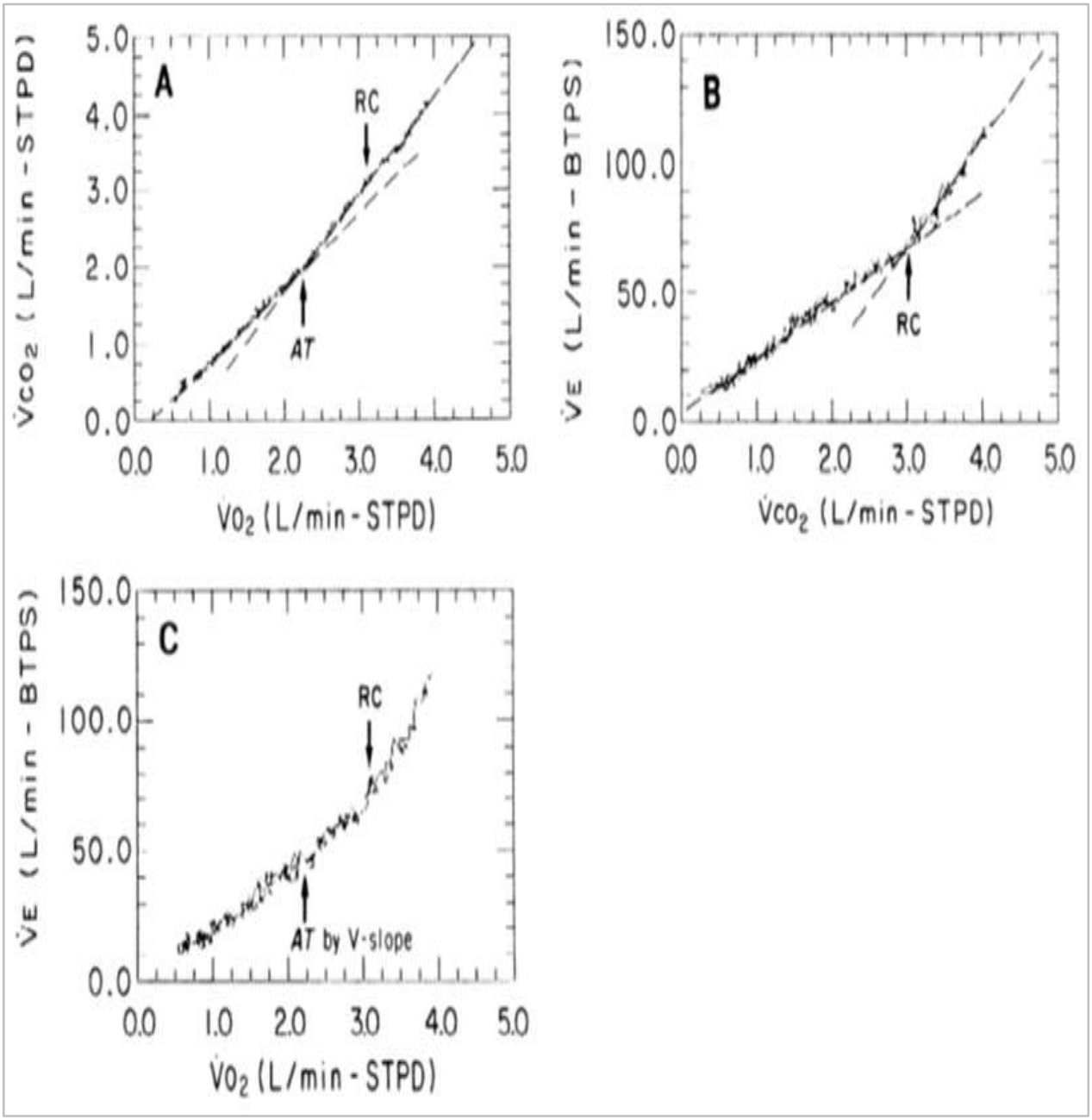


Fig1_ **A:** CO_2 production (VCO_2) vs. O_2 uptake (VO_2) from Fig. 1 (*subject 3*), showing regression lines for detecting inflection point (AT point). **B:** Minute ventilation (VE) vs. VCO_2 for *subject 3*, showing regression lines for detecting inflection point (RC point). **C:** VE vs. VO_2 for *subject 3*, showing AT and RC points derived separately by analysing A and B (Beaver et al., 1986).

Figure 2 - Maximum oxygen consumption

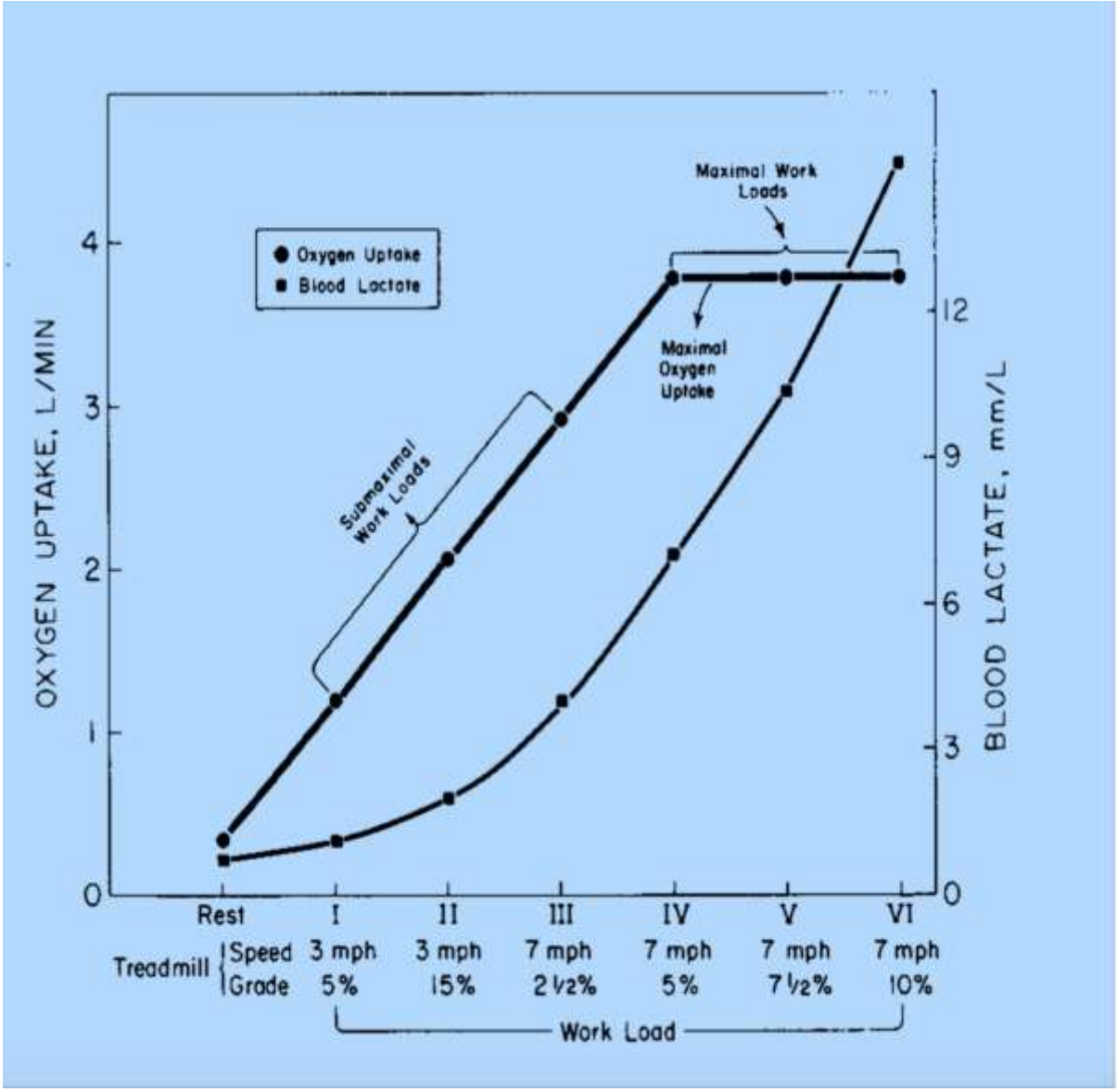


Fig2_Determination of maximal oxygen uptake (Mitchell and Blomqvist, 1971)

In practical terms for the athlete, when reaching AT, the athlete will experience increased ventilation and a sensation of "heavy legs". Furthermore, at RCT the athlete will experience hyperventilation relative to VO_2 and intense fatigue. As the athlete reaches the RCT point they will be increasingly closer to VO_{2max} (Meyer et al., 2004). At VO_2max, the metabolic demand has surpassed the body's ability to deliver sufficient oxygen to the exercising muscles. Meeting the metabolic demands of the task causes the athlete to fatigue leading to termination of the exercise test (Meyer et al., 2004). In addition to VO_2max testing, the endurance capacity test (ECT) is a commonly used performance test. The ECT is used as a

tool to assess the athlete's adaptation to intense continuous exercise (Burgomaster et al., 2005; DiMenna et al., 2009).

In response to exercise, sub-cellular processes manifest in the form of gene expression changes which involves the transcription of DNA (Deoxyribonucleic acid) to RNA (Ribonucleic acid), RNA processing to produce mRNA (Messenger Ribonucleic acid) and the subsequent translation of the mRNA into proteins (Orphanides & Reinberg, 2002). Genes are DNA coding sequences that are part of the entire genome (Orphanides & Reinberg, 2002; Snustad & Simmons, 2006). A gene is a sophisticated controlled DNA sequence that can result in the expression of a protein. Gene expression is carried out through an active regulatory process (Snustad & Simmons, 2006) for which a combination of cellular genes are transcribed or/and translated depending on the biological conditions or requirements of the cell (see Figure 3). Generation of mRNA from DNA requires the binding of transcription factors on promoter DNA regions present upstream of the gene starting point of transcription. Several transcription factors recruit the machinery required for transcription to occur. RNA polymerase enzymes are recruited upstream of the gene and will mediate the transcription of the template DNA to produce RNA molecules (Clancy & Brown, 2008).

Several types of RNA exist including mainly mRNA, ribosomal RNA (rRNA) and transfer RNA (tRNA). rRNA, mRNA and tRNA are produced through the actions of RNA polymerase I, II and III respectively (Clancy & Brown, 2008). All three types of transcribed RNA are essential in the translational process post transcription of mRNA. Other types of RNA also exist such as micro-RNA (miRNA) that can influence the expression of mRNA into proteins (Clancy & Brown, 2008).

Figure 3 - The process of transcription and translation

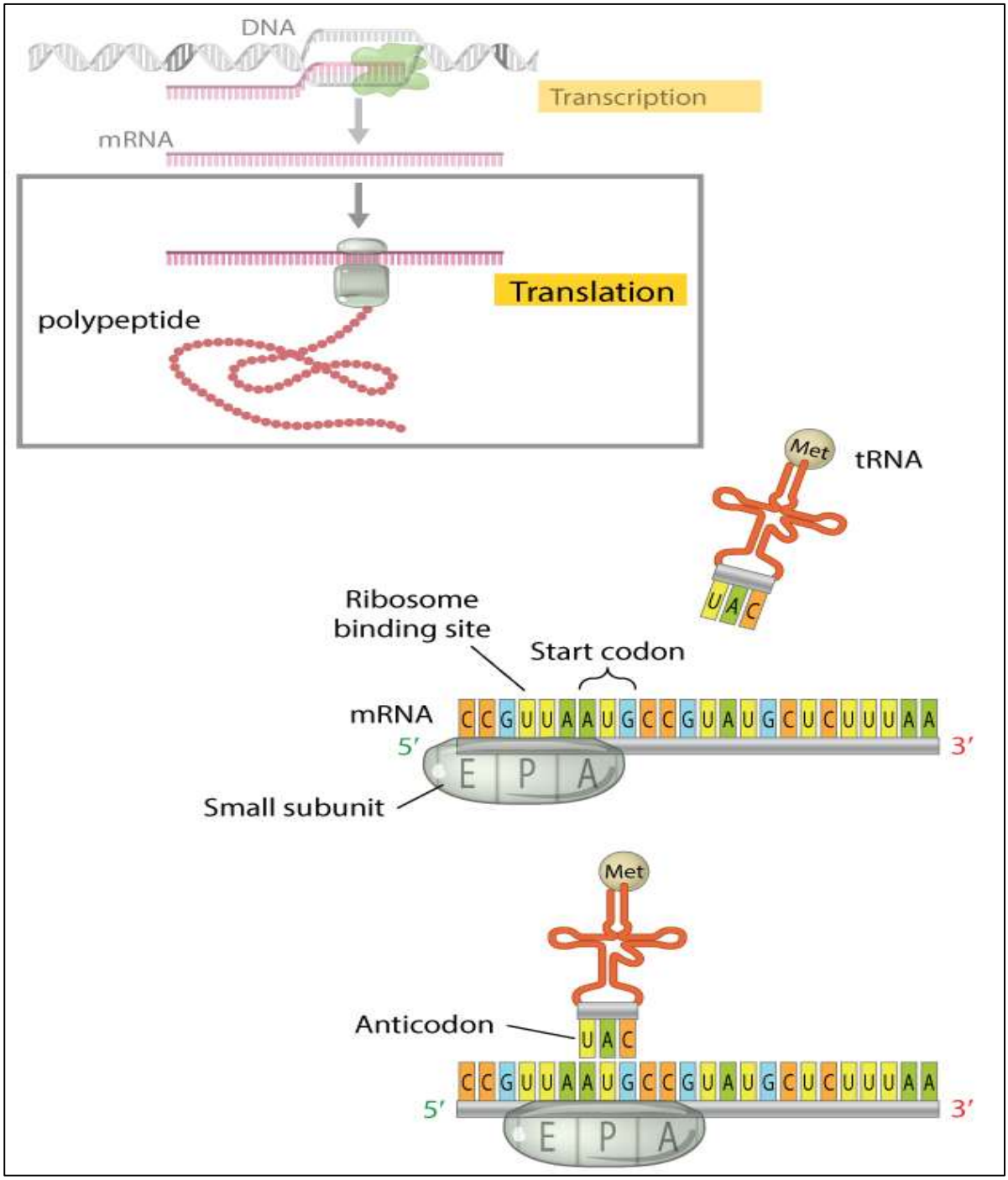


Fig3_The expression of a gene is through the processes of transcription and translation. DNA is used as a template by the enzyme RNA polymerase in order to produce a pre-mRNA transcript. The produced pre-mRNA transcript is then used to create a mature mRNA molecule, which can be used in translation when building the protein molecule (Clancy & Brown, 2008).

Adaptation to exercise starts with expression of particular genes at the muscle tissue level; this in turn leads to alterations in the physiological responses to performance (Zeibig et al., 2005). Well trained endurance athletes show alterations in gene expression related to muscle contraction, structure and development, energy metabolism, mitochondrial proteins, immune response, cell cycle and proliferation, transporters and angiogenesis (Stepto et al., 2009). Furthermore, expression of these genes is linked to phenotypic changes in VO_2max, capillary density, oxidative activity and plasma volume (Stepto et al., 2009) suggesting that traditional training monitoring methods be investigated in comparison with training-induced gene expression changes to help advance athletes in their sport. While trained individuals already possess the mentioned phenotypic adaptations, simply increasing the volume of their sub-maximal training will not generate further performance gains (Laursen & Jenkins, 2002). Training monitoring is a useful tool to assess if the athlete has reached a plateau and needs an alternative or supplementary training method to further stimulate performance. Furthermore, by assessing these changes through gene expression, a potential plateau in performance may be discovered before performance is negatively altered.

When investigating the changes in muscle tissue, the use of muscle biopsies is essential (Zeibig et al., 2005). When performing a muscle biopsy, the overlying skin is anesthetised, an incision is made and the sample is extracted through the incision with a 5-mm Bergstrom needle (Zeibig et al., 2005). It is clear that muscle biopsy is an invasive procedure and a non-invasive alternative should be pursued for further research. Furthermore, gene expression analysis in skeletal muscle cells has been reported to reflect the systemic processes occurring in human white blood cells (WBC) (Zeibig et al., 2005). WBC responses to stimuli can provide a certain understanding of other types of body cells, which are not necessarily easily accessible to researchers. The disadvantages in using WBCs are vast, such as determining the complex transcriptome expression associated with their numerous intrinsic functions including immune regulations and responses. However, these cells are relatively easily accessible, in abundance, and known to react to physiological stimuli and therefore could be considered as an alternative source site to detect and investigate gene expression changes. However, research is greatly lacking in this area.

Interval and intermittent exercise are two types of training strategies that are both based on repeated bouts of exercise and rest in an alternate fashion (i.e. the interval principle) (Sabapathy et al., 2004; Casas, 2008), but can be used for different applications (Casas, 2008). The difference between the interval and intermittent training methods lies in the intensity and duration of the work as well as the intensity and duration of the rest. For instance, intermittent training uses short and high intensity (100-150 % of maximal aerobic velocity) work periods, lasting up to one minute, followed by very light to passive recovery (Casas, 2008). Intermittent training is also referred to in the literature as sprint interval training (SIT). However, interval training is carried out with alternating intensities based on lactate threshold and VO_2max, with longer work periods lasting up to five minutes (Casas, 2008). Both of these training principles reflect a flexible training approach, providing a greater workload with less fatigue and can be manipulated to suit several types of training goals (Astrand et al., 1960b; Tjønna et al., 2008).

Historically interval training can be traced back as far as 1910 (Bourne, 2008). This is where two Finnish gold medallists, Hannes Kolehmainen and Paavo Nurmi, used interval training when preparing for their Olympic performances (Bourne, 2008). During the 1930s, Fartlek or "speed play" was developed by a Swedish coach (Bourne, 2008) which involved different forms of interval training where athletes alternated between fast/slow, fast/medium and medium/slow pace during long runs (Bourne, 2008). However, it was not until German coach Woldemar Gerschler saw the opportunity to include more speed work in a training session that interval training reached its recent popular characterization (Bourne, 2008). Furthermore, although interval training remained an important component of the training program for many types of athletes, it was only used as one part of a periodised program, usually consisting of high volume training (Bourne, 2008). In 1996, a study by Izumi Tabata and colleagues, investigated the variables of interval training. Tabata et al. (1997) found that athletes could achieve improvements in VO_2max with high intensity interval training (HIT) and that HIT had benefits for the anaerobic metabolic system which steady-state aerobic exercise alone could not produce. The same authors showed that less time was required for HIT to produce these results (Tabata et al., 1997).

The increased popularity of HIT has mainly been due to its efficiency in achieving the athletes desired performance gains. Recently however, gene expression and phenotypic performance changes that resemble those associated with endurance training have gained a new scientific interest. It has been suggested that HIT may resemble the prototypical physical activity regime that our genome is adapted to (O'Keefe et al., 2011). Since the beginning of the human race when hunting and gathering was the way of survival, physical activity levels necessary to cover distances of 10 kilometres at 1.5 – 3 km/hour on average per day (with an addition of 1 – 2 km per day covered at high intensity in rapid pursuits) have been shown to be the norm in hunters and gatherers (O'Keefe et al., 2011). Interestingly, these strenuous pursuits could last for more than an hour with continuous burst of 20-30 seconds sprints to capture monkeys fleeing from tree to tree (O'Keefe et al., 2011). This pattern approximates that undertaken in HIT and explains why HIT appears to be a highly efficient training method that our genome responds well to.

Any daily exercise alters the expression of the human genome, resulting in immediate but temporary improvements in parameters such as cardiovascular, musculoskeletal, pulmonary physiology, glucose and lipid metabolism, blood pressure, autonomic balance, mood, sleep quality and immunity (Laursen & Jenkins, 2002; Ziemba et al., 2003). HIT is responsible for physiological changes similar to endurance exercise but without the high volume workload of traditional training techniques. Consequently, HIT is a very important training method, which is capable of improving performance.

Interestingly, optimal frequencies of interval or intermittent training sessions are equivocal. However, there is some evidence suggesting that less frequent and longer duration training programs are superior to higher frequency and shorter duration training programs as the latter generate a significant increase in fatigue (Pollock et al., 1969; Fox et al., 1975). As the intensity, frequency and duration of interval training influence VO_2 differently, the length of the work-recovery periods and the intensity of the work bouts depend on the goal of the session (Casas, 2008). For instance, short and intense bouts will favour anaerobic metabolism. However, when the work interval increases in duration, there are significant elevations in venous and muscle lactate, heart rate (HR) and oxygen uptake (VO_2) while pH and PCO_2 decrease (Morris et al., 2003). At this point aerobic metabolic pathways are

predominantly being used. Interestingly, HIT performance research in cyclists has shown that repeated sprint interval training (SIT) may be equally as effective as more traditional HIT programs aimed at improving endurance performance (Laursen et al., 2002).

Not much is known in regard to prescription of the optimal HIT protocol for any athlete and there is an obvious deficit in the literature with regards to the ideal "regime" of HIT needed to produce maximal adaptational responses in endurance athletes (Laursen & Jenkins, 2002). An exercise regime is a combination of frequency, duration, intensity and recovery (Kesaniemi et al., 2001; Laursen & Jenkins, 2002) and a study by Dalleck et al. (2010) stated that a regime-relationship between the frequency of HIT and the magnitude of lactate and VO_2max improvement does exist. In this study, the investigators found that two days of HIT per week was superior to one day at modifying the lactate threshold (Dalleck et al., 2010). However, the regime-response relationship of HIT to VO_2max and other performance markers is not easily accessible. Further research is required to observe gene expression changes in response to HIT in comparison to traditional training. Such knowledge will pinpoint key markers of performance that can be used to monitor training progress.

In an effort to evaluate and monitor the maximal capacity for anaerobic glycolysis, several short-term anaerobic power tests have been developed. Like other performance tests, anaerobic power tests should involve the specific muscles used in a particular sport, such as the Wingate test when assessing cycling anaerobic power (Bar-Or, 1987; Powers & Howley, 2007). The Wingate test was developed at the Wingate institute in Israel and is a 30 second, maximal effort cycling test (Bar-Or, 1987; Powers & Howley, 2007). The Wingate test has been designed to determine both peak anaerobic power and mean power output and has been shown to be highly reproducible (Inbar & Bar-Or, 1986; Bar-Or, 1987; Minahan et al., 2007; Powers & Howley, 2007).

In addition to increases in performance markers such as VO_2max after HIT in endurance trained athletes, there is a significant amount of literature investigating mechanistic changes at the tissue level as a consequence of gene expression (Burgomaster et al., 2005; Burgomaster et al., 2006; Gibala et al., 2006; Burgomaster et al., 2007; Burgomaster et al., 2008; Rakobowchuk et al., 2008; Gibala et al., 2009; Little et al., 2010). It is therefore

important that the measurement of cellular and molecular responses to training in endurance athletes be studied. One outcome of these complex processes is a change or adaptation at the muscle tissue level (Ross & Leveritt, 2001) and analysis of gene expression from muscle samples may reveal how athletes are responding to training.

However, as sampling muscle tissue is an invasive type of assessment, it has been shown that the alternative use of white blood cells can be suitable to reflect the gene expression alterations occurring in muscle tissue (Maisel et al., 1990; Connolly et al., 2004; Zeibig et al., 2005; Zieker et al., 2005a; Zieker et al., 2005b; Büttner et al., 2007; Radom-Aizik et al., 2008). For this current investigation, a less invasive approach was carried out using white blood cells. These cells present an important source for sampling the molecular changes associated with exercise and present a potential addition to conventional physiological testing and monitoring, such as VO_2max, peak power tests, endurance capacity tests and lactate threshold tests for athletes in sport (Connolly et al., 2004; Zeibig et al., 2005; Büttner et al., 2007).

Whilst there is increasing development into the field of athletic training monitoring in endurance athletes, balancing training regime and volume with recovery and adaptation continues to present an important challenge, due to the extensive physical performance demands of endurance athletes (Rhea et al., 2003). This challenge should be targeted through research combining gene expression studies, in accessible tissue such as white blood cells, with traditional physiological tests. This would allow researchers to discover markers and pathways that can show or predict endurance performance changes and allow the development of individualised training programs which maximise the training impact for each individual athlete and prevent overtraining injuries, resulting in the improved performance of athletes and higher levels of success in sporting events.

1.1 Aims and hypotheses

The aim of the proposed study was to investigate high intensity exercise and its effect on physiological and biochemical adaptation and regulation in relation to athletic performance. HIT has been extensively used for years in many types of training prescription, particularly

for its drastic effect on the metabolic system. However, the regimes of work: rest ratio, interval numbers and session numbers per week needed to achieve these adaptations are still unclear. This study will endeavour to investigate if metabolic changes caused by HIT are regime-dependent (testing two regimes: low-volume/high-frequency and high-volume/low-frequency). Furthermore, this study will investigate if as little as nine bouts of 30-second sprints per week for two weeks will generate endurance performance changes.

> **Aim 1:** To determine whether 18 repetitions of 30s bouts of all-out sprints over two weeks are sufficient to significantly improve physiological changes in relation to performance.

> **Aim 2:** To determine which exercise delivery method (low-volume/high-frequency or high-volume/low-frequency) creates the most favourable physiological changes in relation to performance; as determined by the endurance capacity test, anaerobic threshold (via VO_2max test), and the Wingate test.

In order to achieve this, twenty-six healthy trained male cyclists were randomly allocated into one of three groups, control (without training), group 1 (with low-volume/high-frequency training), and group 2 (with high-volume/low-frequency training). Group 1 (with low-volume/high-frequency training) underwent three repetitions of 30s all-out sprints, with four min recovery between each repetition on six different occasions over two weeks. Group 2 (with high-volume/low-frequency training) carried out nine repetitions of 30s all-out sprints, with four min recovery between each repetition on two occasions over two weeks. The control group underwent no training intervention over the two-week training period.

<u>Before and after training intervention, each subject underwent</u>:

1) Maximum oxygen consumption test via a VO_2max test
2) Anaerobic threshold test via a VO_2max test
3) Wingate test
4) Endurance capacity test.

Hypothesis 1: Two weeks of high intensity sprint interval training (group 1 and 2) will improve all selected outcome performance measures relative to the control group.

Hypothesis 2: Of the two training regimes (high frequency / low frequency), group 2 (low frequency / high volume, with 9 repetitions 1 time per week) will induce a higher level of maximum oxygen uptake as measured by the VO_2max test.

Hypothesis 3: Of the two training regimes (high frequency / low frequency), group 1 (high frequency / low volume, with 3 repetitions 3 times per week) will induce a higher increase in anaerobic thresholds as measured by the VO_2max test.

Hypothesis 4: Of the two training regimes (high frequency / low frequency), group 1 (high frequency / low volume, with 3 repetitions 3 times per week) will induce a higher increase in power as determined by the Wingate test.

Hypothesis 5: Of the two training regimes (high frequency / low frequency), group 2 (low frequency / high volume, with 9 repetitions 1 time per week) will result in a longer time to fatigue (and therefore increased endurance capacity) as measured by the endurance capacity test.

Additionally, to achieve a full understanding of the biochemical adaptations and regulation associated with high intensity cycling, an examination of genes in-vivo (using human WBCs) will be performed. The focus will be on those genes found through genome-wide microarray, which have a biological basis of the regulation and adaptation resulting from repeated high intensity exercise. The aim is also to investigate the changes in expression levels in response to varying training strategies (with concurrent effects of frequency of sessions per week and number of bouts per session). Peripheral blood is an accessible source of cells in which to investigate circulating leucocytes as “scouts”, which continuously maintain a comprehensive surveillance of the body for signs of infection or other threats (Büttner et al., 2007). It has also been shown through previous studies that human white blood cells may prove to be a useful area of investigation when attempting to determine the mechanism that links physical activity with health. The measurement values will be expressed as fold changes,

reflecting process where information from a gene is used in the synthesis of a functional gene product.

Aim 3: To identify a set of specific genes whose expression is significantly affected by SIT (global changes in gene expression after training).

Aim 4: To identify whether there are any significant differences in gene expression between the two training methods (low-volume/high-frequency or high-volume/low-frequency).

Aim 5: To identify a set of genes that reflect and/or are highly correlated with the identified performance changes from the endurance capacity test, the anaerobic threshold (via VO_2max test), and the Wingate test.

Twenty-six healthy trained male cyclists were recruited and randomly allocated into one of three groups (control, group 1 & group 2). The subjects in the **control group** came to Bond University and donated blood on two occasions (before and after the two-week training intervention). Subjects in this group did not undertake any training intervention. The subjects in **group 1** were asked to visit Bond University, during which they underwent three repetitions of 30s all out sprints, with four min recovery between repetitions on six different occasions over two weeks. The subjects donated blood on several occasions throughout the training intervention. **Group 2** was asked to visit Bond University in order to carry out nine repetitions of 30s all out sprints, with four min recovery between reps on two occasions over two weeks. Each subject donated blood on several occasions throughout the training intervention.

Hypothesis 6: A large number of genes will show changes in gene expression due to SIT. However, a specific set of genes will reflect the acute and delayed response of this mode of training.

Chapter 2 – Review of the Literature

2.1 Exercise Physiology

A basal level of oxygen is required to sustain human life, while during exercise the demands for energy (ATP) are greater and the increase in skeletal muscle work creates a rise in the demand for oxygen (O_2). As a result, cellular respiration must increase and the circulatory, pulmonary and especially the cardiovascular systems all undergo alterations. The increased demand for oxygen is met by an increase in pulmonary oxygen uptake (VO_2) (Fagard, 2003; Klasnja et al., 2013), while the cardiovascular system is responsible for transporting oxygen-rich blood from the lungs to the skeletal muscle (Klasnja et al., 2013). Only through adaptations can the cardiovascular system sufficiently meet the oxygen demands of exercising skeletal muscle (Klasnja et al., 2013). This can be achieved through adaptations that increase cardiac output, the amount of blood pumped by the left ventricle per minute, which increases up to 5 - 6 fold during a maximal exercise effort (Weiner & Baggish, 2012). For instance, long-term aerobic training increases the heart's mass and volume, resulting in an increase of left ventricular end diastolic volume during rest and exercise (Morganroth et al., 1975; George et al., 1999; Pluim et al., 2000; Venckunas et al., 2008; Pelliccia et al., 2012). Furthermore, the cardiac muscle typically adapts to increased workload through moderate hypertrophy attributed to longitudinal myocardial cell enlargement, which occurs independent of age (Oakley, 2001), although left ventricular enlargement, which is characterised by an increased cavity size combined with a thickening of the ventricular walls, returns to a pre-training state when training ceases (Oakley, 2001). Long-term exercise also alters the contractile properties of cardiac muscle fibres by increasing their sensitivity to calcium (Ca^{2+}) activation, causing changes in the force-length relationship and resulting in increased power output (Pluim et al., 2000; Wisløff et al., 2001). In trained muscle, the RNA content increases and protein synthesis accelerates. This leads to a thickening of individual myofibrils and an increase in contractile filaments (Sjöblom et al., 2008; Weiner & Baggish, 2012).

While myocardial structural and dimensional adaptations to regular exercise generally reflect specific training demands (George et al., 1999; Weiner & Baggish, 2012), an increase

in skeletal muscle work is essential during all forms of training in which there is a direct relationship between intensity and the body's demand for oxygen (Klasnja et al., 2013). Furthermore, when training is chronically applied morphological changes in the heart follow, including increases in left ventricular chamber size, wall thickness and mass (Pluim et al., 2000; Oakley, 2001; Klasnja et al., 2013). The morphological changes to the heart, specifically the increase in left ventricular mass as a response to exercise, is named "athlete's heart" and includes a strength trained heart and an endurance trained heart (Morganroth et al., 1975; Pluim et al., 2000; Oakley, 2001). Furthermore, endurance trained athletes who are involved in highly dynamic sports (e.g. running) mainly develop an increased left ventricular chamber size with a proportional increase in wall thickness as a response to the high demand of cardiac output related to their sport (Morganroth et al., 1975; Pluim et al., 2000). In contrast, athletes that are trained in strength and power sports (i.e. weightlifting and sprints) have predominantly developed an increased left ventricular wall thickness and an unchanged left ventricular chamber size, due to the pressure overload from the high systemic arterial pressure accompanying their sport (Morganroth et al., 1975; Pluim et al., 2000). Furthermore, endurance trained athletes exhibit an eccentric left ventricular hypertrophy, while strength and power trained athletes demonstrate concentric left ventricular hypertrophy (Morganroth et al., 1975; Pluim et al., 2000). In fact, with prolonged exercise training, stroke volume adapts by significantly increasing both at rest as well as during exercise (Weiner & Baggish, 2012). This in turn increases cardiac output as it is the product of stroke volume and heart rate.

The relationship between blood volume and maximal oxygen consumption has been confirmed in the literature (Fortney et al., 1988; Yoshida et al., 1997). Moreover, a higher total blood volume has been reported to exist in athletes compared to non-athletes (Brooks et al., 2004). Additionally, plasma volume, erythrocyte volume and total haemoglobin content are also higher in athletes (Brooks et al., 2004). Independent of physical aptitude, it has been established that blood volume and haemoglobin concentration serve an important role in oxygen transport during exercise (Kawabata et al., 2004). Functionally, elevated blood volume increases cardiac output through facilitating venous return (Brooks et al., 2004). Since plasma volume is a major constituent of blood volume, exercise-induced changes in this component of whole blood have been a consideration in exercise research.

Studies have shown that an acute decrease in plasma volume is directly related to exercise intensity and duration (Galbo et al., 1976; Wilkerson et al., 1977). Consequently, an exercise-induced decrease in plasma volume tends to create a fall in blood volume (Buchheit et al., 2009). For example, one study investigated the changes in blood volume, red cell volume and plasma volume during sub-maximal treadmill exercise at 30, 45, 60, 75 and 90% VO_2max (Wilkerson et al., 1977). This study showed that a decrease in plasma volume was directly related to exercise intensity (Wilkerson et al., 1977). Another study found that plasma volume decreased significantly during exercise, from an average of 2685.4 mL, by 19.6% or 532.4 mL (Kawabata et al., 2004). It was suggested that these results were a product of the osmotic movement of water from the intravascular space into active muscle (Kawabata et al., 2004; Karamizak et al., 1994). Additionally, increases of 2.5 mm Hg of interstitial fluid pressure in contracting muscles have been reported after maximal exercise versus no change in resting muscle (Mohsenin & Gonzalez, 1984). However, fluid volumes are controlled by several factors such as; the renal pressure diuresis mechanism, vasopressin stimulation of water-reabsorption by the kidneys, control of water reabsorption by thirst, and stimulation of sodium reabsorption and potassium secretion in the renal tubules by aldosterone (Brooks et al., 2004). It has been shown that plasma volume decreases approximately 10% during prolonged exercise (Brooks et al., 2004).

This reduction in plasma volume places an increased load on the circulatory system during continuous exercise. Fortunately, adaptation to chronic exercise results in an increase in blood volume, which in turn increases stroke volume, blood flow and the circulatory capacity (Brooks et al., 2004; McArdle et al., 2000). Furthermore, mean arterial blood pressure, which is the product of cardiac output and the peripheral vasculature resistance to blood flow (i.e. total peripheral resistance) (McArdle et al., 2000), rises during exercise in order to maintain blood flow to critical areas such as the heart and brain while at the same time meeting the requirement of the working muscles (Brooks et al., 2004; McArdle et al., 2000). In fact, in top-level cyclists a systolic blood pressure reading of >200 mm Hg can be found during maximal exercise testing on a cycle ergometer (Pluim et al., 2000). The extreme pressure and volume load found in the hearts of cyclists may explain why the largest increases in the left ventricular dimensions and left ventricular wall thickens has been found in this type of athlete (Pluim et al., 2000).

2.2 Energy systems and adaptations to exercise

In order for muscle contraction to occur, energy (in the form of adenosine triphosphate) is delivered via anaerobic and aerobic metabolic pathways. Adenosine triphosphate (ATP) is a nucleoside triphosphate that breaks down to release the energy responsible for muscle contraction, however it cannot be stored and requires continuous regeneration. Under conditions of exercise the body utilizes three energy systems in order to meet the high demand of the body's ATP requirements. Two of these systems (ATP-PC and glycolysis) do not require oxygen for their operation. The third energy system of the body, which utilizes the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, is referred to as the "aerobic" energy system. Energy transduction in this system is dependent on the presence of oxygen (Brooks et al., 2004; Plowman & Smith, 2013). Energy metabolism can use diverse types of molecules including carbohydrates, lipids and protein. Metabolic pathways are characterised as either anabolic (glycogen for carbohydrate storage, gluconeogenesis, lipid synthesis for fat storage and protein anabolism) or catabolic (glycogenolysis, lipid beta oxidation and protein catabolism). In general, during exercise carbohydrates and lipids are the preferred energy sources. Carbohydrates, such as glucose, can be catabolised by the cell during glycolysis under anaerobic conditions to provide acetyl CoA. Under aerobic conditions, oxygen is used as the final acceptor of electrons and enables further metabolic reactions downstream of pyruvate formation. Energy derived from the catabolism of lipids via β-oxidation generates acetyl CoA substrates that enter these aerobic pathways and are therefore dependent on the presence of oxygen. Limitations of energy supply and saturated metabolic capacities results in decreased performance due to fatigue that is characterised by a drop in ATP generation and increases in by-product accumulation and cellular stress.

During sprint interval training, fatigue (failure to repeat performance across repetitions) is displayed by a decline in the peak/mean power output or the speed of the interval (i.e. the time to cover a fixed distance is increased) (Glaister, 2005; Bishop et al., 2011). A limitation of energy supply and metabolite accumulation has been proposed as the cause of fatigue (Racinais et al., 2007; Mendez-Villanueva et al., 2007). However, there are certain training intervention strategies that have been proposed to decrease the limiting factors and improve sprint repeatability capacity (Bishop et al., 2011: Girard et al., 2011).

2.2.1 Metabolic pathways - ATP -Phosphocreatine Pathway

The fastest biochemical ATP production is generated by the ATP-PC system (Gastin, 2001). This system involves the transfer from phosphocreatine (PC) of a phosphate group and its energy bond to ADP resulting in the formation of ATP as displayed in the equation below (Gastin, 2001; Robergs, 2001; Powers & Howley, 2007).

$$PC + ADP + H^{+} \xrightarrow[\text{Creatine kinase}]{} ATP + Creatine$$

Creatine kinase is the enzyme responsible for catalysing this reaction and can renew the supply of ATP molecules as they are utilised at the onset of exercise (Brooks et al., 2004; Gastin, 2001). Conversely, muscle tissue is only capable of storing a limited amount of PC and as a result, the creation of ATP through this reaction is limited (Brooks et al., 2004; Gastin, 2001). These restricted stores of ATP and PC are called the "ATP-PC system", which is the source of energy during the onset of exercise and during high intensity exercise lasting less than five seconds (Gastin, 2001; Powers & Howley, 2007). The ATP-PC system plays an important role during exercise such as rapid weight lifting, 50-meter sprints and a long jump.

Propositions have been made regarding phosphocreatine and the fact that the ability to synthesize this molecule might be an important element in the repetition of sprint performance (Bogdanis et al., 1996; Dawson et al., 1997). This is based on the fact that, during repeated sprint training, recovery times between bouts are brief which will cause phosphocreatine stores to only partly recover (Bogdanis et al., 1995; Bogdanis et al., 1996). Interestingly, it has been suggested that improvements in repeated sprint ability and resynthesis of phosphocreatine, is strongly related to recovery between bouts (Bogdanis et al., 1995; Bogdanis et al., 1996). Furthermore, it has been reported that oxidative metabolism is critical for the resynthesis of phosphocreatine during the recovery period between high-intensity bouts (Haseler et al., 1999). This suggestion indicates that individuals with a high VO_2max or LT threshold are able to resynthesise phosphocreatine faster during the recovery period and in turn have higher repeated sprint ability (McCully et al., 1989; McCully et al., 1992; Yoshida et al., 1997; Yoshida & Watari, 1993; Bogdanis et al., 1996). However, as sprint interval training has been stated to increase aerobic fitness and as

aerobic fitness is required to resynthesise phosphocreatine, more research is needed to make clear which training intensity is superior to create optimal performance.

2.2.2 Metabolic pathways - Glycolysis

Another metabolic pathway that has a rapid capacity to produce ATP without the requirement for oxygen is glycolysis (see figure 4). This process involves a series of enzymatically catalysed coupled reactions transferring bond energy from glucose to re-join P_i to ADP (von Duvillard, 2001; Gastin, 2001; Powers & Howley, 2007). Glycolysis occurs in the sarcoplasm of the muscle cell and produces ATP and pyruvate or lactate (von Duvillard, 2001; Powers & Howley, 2007). The glycolytic pathway has two phases. Phase "one" is the energy investment phase where 2 moles of ATP are consumed for each mole of glucose (Robergs et al., 2004). A phosphate is added to glucose yielding glucose-6-phosphate that is subsequently converted into fructose-6-phosphate and in turn fructose-1, 6-bisphosphate that is then cleaved, generating two molecules of glyceraldehyde-3-phosphate. Phase "two", which is the energy 'payoff' phase, harvests four moles of ATP and two moles of NADH from each initial mole of glucose. Moreover, each glyceraldehyde-3-phosphate molecule is oxidized via five enzymatic reactions to yield pyruvate (see Figure 4) (Gastin, 2001; Powers & Howley, 2007).

During SIT, there is a rise in ADP and Pi, which is consistent with a drop in intramuscular phosphocreatine (Crowther et al., 2002). As a result of this, the start of a sprint bout produces a fast activation of anaerobic glycolysis. Consequently, single sprints rely largely on ATP production from anaerobic glycolysis (Gaitanos et al., 1993). However, for subsequent sprints the ATP produced from anaerobic glycolysis decreases considerably (Sahlin & Ren, 1989; Bogdanis et al., 1995). For this reason, it's uncertain whether an increase in the maximal anaerobic glycogenolytic and glycolytic rate will create improvements in sprint interval ability (Gaitanos et al., 1993; Mendez-Villanueva et al., 2008). Interestingly, arguments have been made as to whether training which promotes increases in the supply of ATP from anaerobic glycolysis would have a negative effect on subsequent sprint repeatability. This argument is reflected by the ongoing decrease in performance of subsequent repeated sprints associated with the restriction of ATP synthesis resulting from

the first sprint (Gaitanos et al., 1993). Adversely, subjects with a larger primary sprint performance have been reported to have a greater glycogenolytic rate (Gaitanos et al., 1993). Additionally, initial, final and total sprint performance have been found to have a strong correlation during sprint repeatability tests, thus highlighting the difficulties coming forth while attempting to interpret the various test measures of sprint repeatability (Bishop et al., 2003; Pyne et al., 2008). However, it has been suggested that, in order to improve initial and mean sprint performance, an increase in the anaerobic involvement is advantageous when improving final sprint performance. Consequently, further research is needed to examine the association between sprint repeatability and anaerobic ATP production (Parra et al., 2000; Jacobs et al., 1987).

Figure 4 - Anaerobic metabolism of glucose – Glycolysis

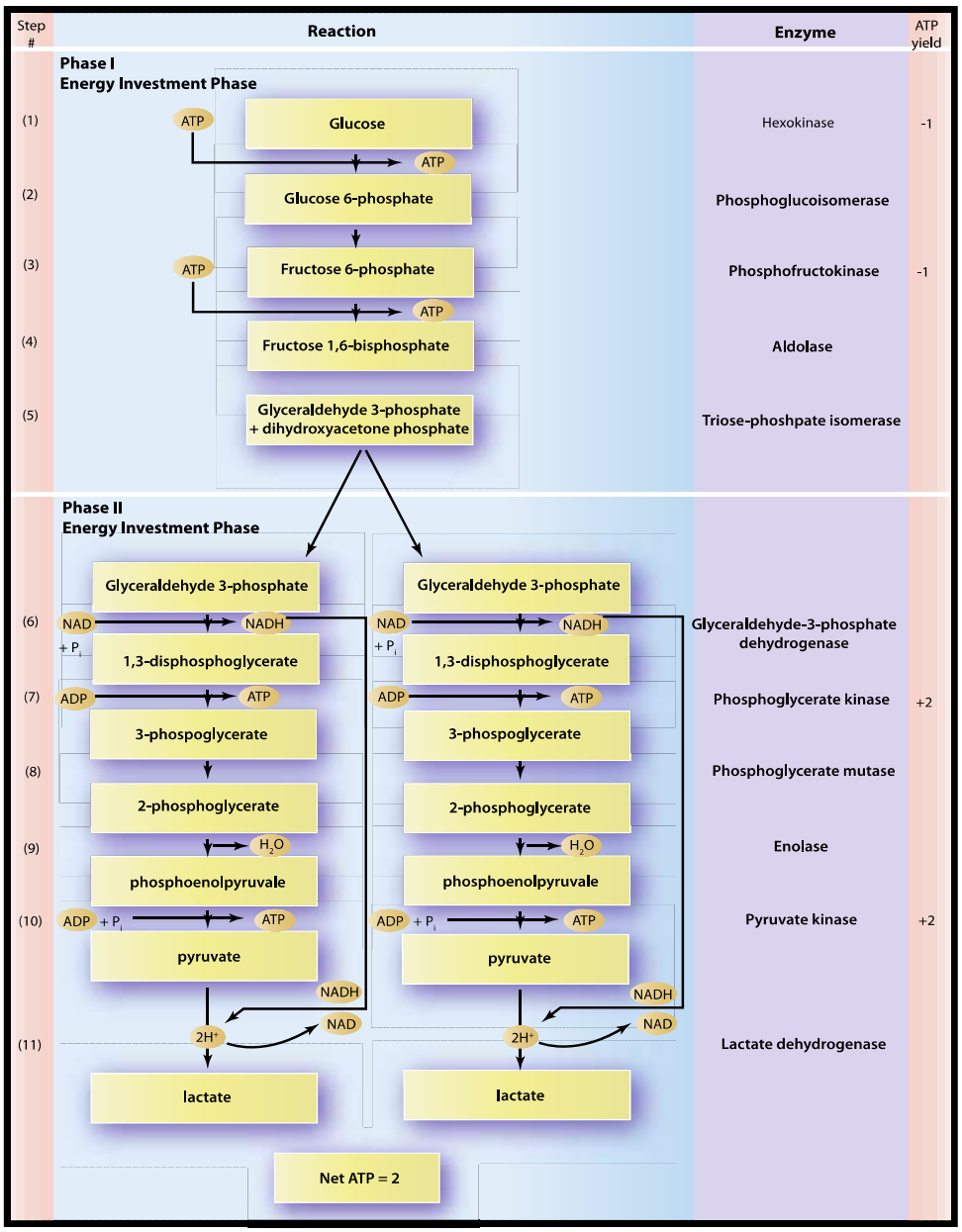


Fig4_ Summary of the anaerobic metabolism of glucose (Powers & Howley, 2007). Five enzymatic reactions are used in phase I of glycolysis requiring the consumption of two ATP to convert one molecule of glucose into two molecules of glyceraldehyde 3 phosphate. In Phase II, a process involving five enzymatic reactions converts two glyceraldehyde 3-phosphate molecules into two pyruvate molecules. Phase II yields four molecules of ATP and two molecules of NADH (donor of electrons to the electron transfer chain of molecules in the inner membrane of mitochondria). In anaerobic conditions, pyruvate is converted to lactate by the enzyme lactate dehydrogenase.

2.2.3 Metabolic pathways - Beta Oxidation

The fatty acid oxidation enzymes are located in the mitochondrial matrix (Kerner & Hoppel, 2000). Fatty acids must be activated for degradation by conjugation with coenzyme A (CoA) in a reaction catalysed by acyl-CoA ligases (thiokinases) (Kerner & Hoppel, 2000). These enzymes are associated with the endoplasmic reticulum and outer mitochondrial membrane and require ATP. Note that ATP is cleaved to Adenosine Mono Phosphate (AMP) and pyrophosphate (PP_i). Cleavage of PP_i to 2 P_i by inorganic pyrophosphatase helps to drive the acylation reaction to completion (Kerner & Hoppel, 2000).

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \rightleftharpoons \text{fatty acyl-CoA} + \text{AMP} + PP_i$$ (Powers & Howley, 2007)

Although fatty acid activation occurs in the cytosol, the molecule has to be transported inside the mitochondrion in order to undergo B-oxidation. That regulated transfer of fatty acyl-CoA is aimed at translocating the acyl groups through both the external and the impermeable inner mitochondrial membranes. The acyl-CoA group is trans-esterified to carnitine in a reaction catalysed by Carnitine acyl transferase I (located on the external surface of the inner mitochondrial membrane) or II (inner surface of the inner mitochondrial membrane) (Kerner & Hoppel, 2000). The acyl-carnitine/carnitine transporter protein transfers acyl carnitine into the mitochondrion as it transfers free carnitine out. Once inside, the acyl group is transferred from carnitine to mitochondrial CoA yielding acyl-CoA. The free carnitine is then transported back out by the carnitine shuttle (Kerner & Hoppel, 2000).

Inside the mitochondrion, acyl-CoAs are acted upon in four reactions that yield one NADH and one $FADH_2$ per acetyl CoA formed from Acyl-CoA (Kerner & Hoppel, 2000). Oxidation of acetyl-CoA in the tricarboxylic acid cycle (TCA-Cycle) generates more NADH and $FADH_2$ carriers. Complete oxidation of one palmitate molecule (16 carbons) generates 129 ATPs (Kerner & Hoppel, 2000). The primary reason that glycogen reserves are essential is that athletes can only slowly convert their body fat stores into energy during exercise. Therefore, when muscle glycogen and blood glucose concentrations are low, the intensity of exercise must be reduced to a level that can be supported by the body's ability to convert body fat into energy (Plowman & Smith, 2013). With endurance training, athletes can markedly increase the rate at which body fat can be oxidized, thus allowing them to exercise longer

before becoming exhausted due to glycogen depletion (Plowman & Smith, 2013; Faria et al., 2005a).

2.2.4 Metabolic pathways - TCA Cycle

The primary function of the TCA Cycle is to remove electrons and hydrogen atoms from various substrates involved in the cycle and transfer their associated energy to the electron transport chain (Plowman & Smith, 2013). As illustrated in figure 5, three molecules of NADH and one molecule of $FADH_2$ are formed during each "turn" of the TCA Cycle (Powers & Howley, 2007). Each NADH molecule will be used to produce 3 molecules of ATP, whereas each $FADH_2$ molecule will produce 2 molecules of ATP during oxidative phosphorylation (Powers & Howley, 2007). In addition to the production of NADH and $FADH_2$, the Krebs cycle results in a formation of an energy-rich compound, guanosine triphosphate (GTP) (Powers & Howley, 2007). GTP is a very high-energy compound that can transfer its phosphate group to ADP in order to form ATP (Powers & Howley, 2007).

Figure 5 - TCA cycle

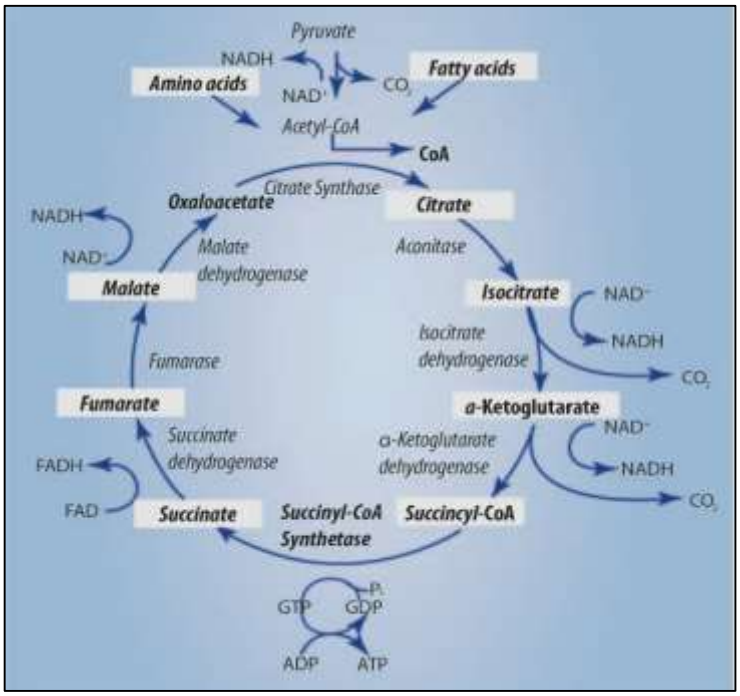


Fig5_Compounds, enzymes, and reactions involved in TCA cycle (Powers & Howley, 2007). A metabolic pathway consisting of eight enzymatic reactions, one cycle of which generates 3 molecules of NADH, 1 molecule $FADH_2$ and 1 molecule of ATP.

2.2.5 Electron Transport Chain

The electron transport chain produces energy aerobically through a mechanism that uses the potential energy available from carrier molecules NADH and $FADH_2$ to rephosphorylate ADP to ATP (Brooks et al., 2004; Weibel et al., 1996). The bulk of the electrons entering the electron transport chain are in the form of the reduced carrier molecules NADH and $FADH_2$ that are formed primarily as a result of the electron transport chain in the mitochondrial matrix (Brooks et al., 2004). In order for NADH and $FADH_2$ to rephosphorylate ADP to ATP, their electrons must be transported along the inner mitochondrial membrane by special shuttle mechanisms (Weibel et al., 1996). At the end of the electron transport chain (Complex IV), oxygen accepts the electrons that are passed along the chain and combines with two hydrogen molecules to form water (Powers & Howley, 2007) (see Figure 6). If oxygen is not available as a final acceptor of electrons, oxidative phosphorylation is inhibited and ATP formation must occur through anaerobic metabolism (Powers & Howley, 2007), a limitation for endurance activity. ATP is formed in the electron transport chain, through chemiosmosis (Weibel et al., 1996). There are three pumps (termed complex I, complex III and complex IV) that move H^+ from the mitochondrial matrix to the intermembrane space (Powers & Howley, 2007). As a result, there is a higher concentration of H^+ within the intermembrane space compared to the matrix and this electro-chemical gradient creates a strong drive for these H^+ to diffuse back into the matrix (Weibel et al., 1996). These H^+ do so via the enzyme complex ATP synthase (Complex V), activating the enzyme in the process and coupling the consumption of oxygen by the ETC to the regeneration of ATP (Weibel et al., 1996).

Independent of the fibre type being used, ATP is the ultimate source of energy for contraction. Muscle contraction is a consequence of actin-myosin cross-bridge formation and release due to myosin-ATPase hydrolysis of ATP (Weibel et al., 1996). Furthermore, high turnover of ATP during contraction consumes the molecule within 1 minute and as the intracellular stores of ATP are small, regeneration must be rapid and continual (Weibel et al., 1996). Exercise intensity controls the contribution of substrates to energy production in the working muscle. As shown in this section, “low” to “moderate” exercise intensity is mainly supported by lipid oxidation. When exercise intensity reaches 50-55% of VO_2max, lipid oxidation declines and carbohydrates are mainly used. Carbohydrates are the most effective

fuel for working muscle and their impact on total fuel oxidation is positively linked to muscle workload. Initially, ATP production from glucose is continued by skeletal muscle glycogenolysis and glycolysis. Later, circulating glucose, continuously made through activated gluconeogenesis, becomes increasingly important. Recent evidence suggests that, among glucose metabolites, lactate has a crucial role as either a direct or indirect energy source for contracting skeletal muscle. Finally, protein input to ATP production is very limited (less than 2%), becoming larger only in the presence of severe muscle glycogen depletion (Weibel et al., 1996).

Figure 6 - Electron transport chain

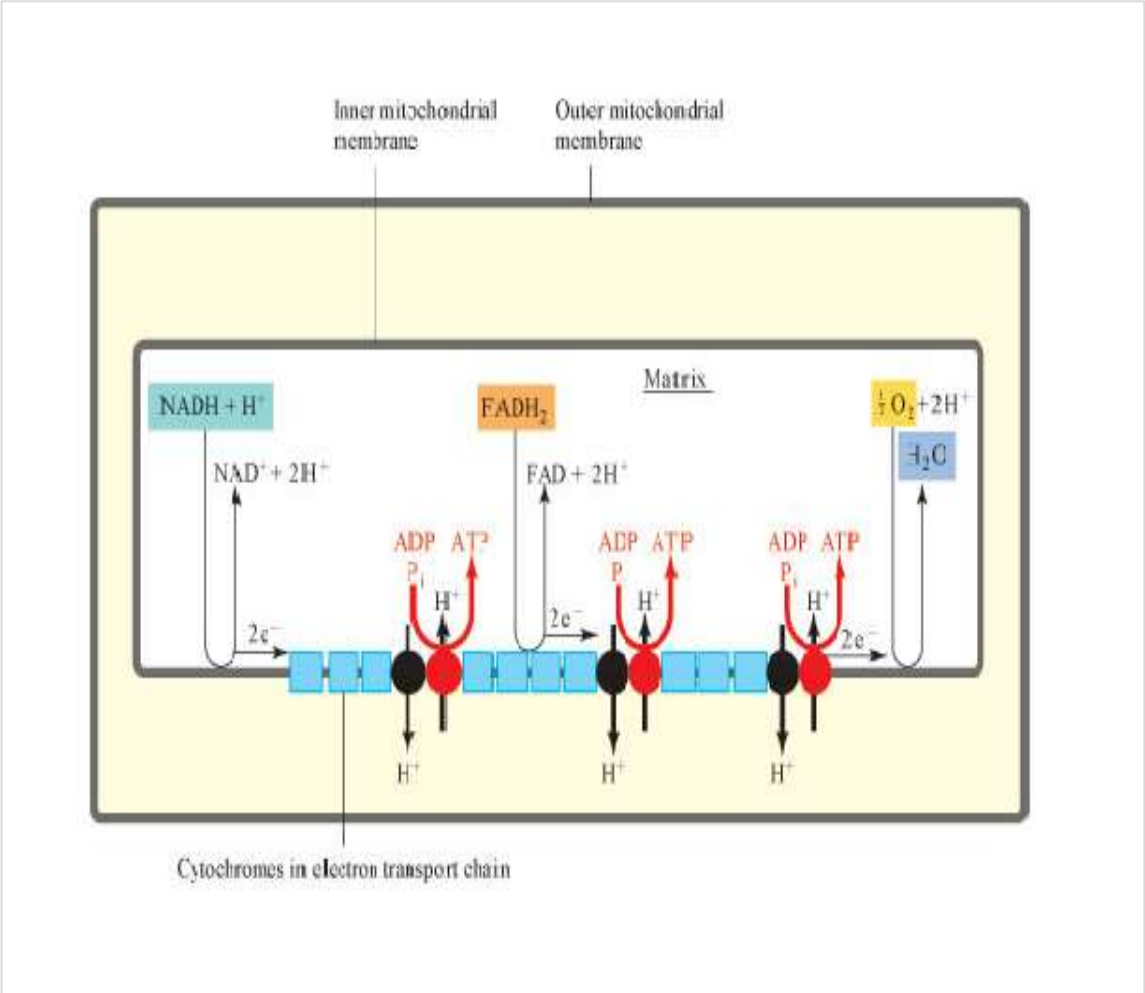


Fig6_The transfer of electrons across the ETC ends with the final acceptor of electrons: the oxygen molecule. ATP is formed during oxidative phosphorylation by the influx of hydrogen ions across the inner mitochondrial membrane to the matrix (Powers & Howley, 2007).

Physiological factors such as higher VO_2max, lactate threshold, mitochondrial respiratory capacity, faster oxygen uptake and accelerated post-sprint re-oxygenation of the muscle can be associated with re-synthesizing ATP and resisting fatigue during sprint intervals (McMahon & Wenger, 1998; Tomlin & Wenger, 2002; Thomas et al., 2004; Bishop et al., 2004; Bishop & Spencer, 2004; Dupont et al., 2005; Rampinini et al., 2009; Buchheit & Ufland, 2011). VO_2max has been widely investigated leading to suggestions that athletes with a greater VO_2max possess a greater ability to resist fatigue during sprint interval training (McMahon & Wenger, 1998; Bishop et al., 2004; Bishop & Spencer, 2004; Rampinini et al., 2009). This is especially true in the later stages of interval training, as subjects should be reaching their VO_2max. This suggests that in order to increase the aerobic contribution when performing repeated sprints, VO_2max improvements are necessary (Bishop & Edge, 2006). Furthermore, it is evident that several physiologists believe the reduction of oxygen levels during training is the factor that motivates the increase in VO_2max (McMahon & Wenger, 1998; Bishop et al., 2004; Bishop & Spencer, 2004; Rampinini et al., 2007). It has been shown that a reduction of oxygen levels in the muscle occurs up to an intensity of 100% of VO_{2max}, a point at which no further increase in intensity will change muscle oxygen levels (MacDougall & Sale, 1981; Daussin et al., 2008). It is then evident in the literature that interval training that approximates VO_2max intensities provokes higher improvements in VO_2max compared to continuous training (Evertsen et al., 2001; Daussin et al., 2008). Additionally, interval training has been shown to concurrently advance other factors such as the phosphocreatine re-synthesis rate and the buffering capacity of the muscle (Edge et al., 2006; Bishop et al., 2004).

2.3 Endurance training

Performing a specific mode of training repeatedly over a period of time causes numerous gene expression, biochemical and physiological changes that result in improved performance in that activity. Endurance can be defined as the capacity to sustain a given velocity or power output for the longest possible time (Jones & Carter, 2000; Laursen & Jenkins, 2002). Performance in endurance events is therefore heavily dependent upon the aerobic resynthesis of ATP which requires an adequate delivery of oxygen to the mitochondrial electron transport chain (Coyle, 1999; Jones & Carter, 2000). Additionally, the adaptations that accompany endurance and LSD training are a result of an increase in muscle energy demand (Booth et al., 1998; Jones & Carter, 2000; Laursen & Jenkins, 2002). Furthermore, by manipulating the frequency, duration and intensity of work together with rest durations, oxygen delivery to muscle as well as metabolic pathways within the muscle cells can be manipulated (Jones & Carter, 2000; Hawley, 2002a; Laursen & Jenkins, 2002). It is these characteristics of a particular training program that create adaptation at the cellular level that ultimately produces changes at the systemic level (Laursen & Jenkins, 2002; Laursen et al., 2005).

In previously untrained individuals, endurance training increases the delivery of oxygen to the exercising muscles (central adaptations) and increases the utilisation of oxygen by the working muscles (peripheral adaptations), creating an adaptational increase in physical work capacity (Green et al., 1990; Green et al., 1991; Jones & Carter, 2000). The central adaptations to endurance training are; lower heart rate, increased stroke volume, cardiac output, blood volume and muscle blood flow during exercise (Green et al., 1990; Spina et al., 1992; Spina, 1999; Jones & Carter, 2000). Furthermore, endurance training creates peripheral changes such as a reduction in glucose and muscle glycogen utilisation, increases in muscle capillary density and mitochondrial volume, as well as lower blood lactate levels during sub-maximal exercise, all created as a consequence of alterations in gene expression (Green et al., 1990; Green et al., 1991; Kiens et al., 1993; Coyle, 1999; Jones & Carter, 2000; Laursen & Jenkins, 2002). However, while central adaptations and increases in physical work capacity occur relatively rapidly (approximately 3 days) (Green et al., 1991; Laursen & Jenkins, 2002), changes in VO_2max, muscle capillary density and mitochondrial volume

require a longer period of continuous training in order to adapt (Hickson et al., 1981; McKenzie et al., 2000). Nevertheless, when endurance training becomes a routine, as it is for athletes, simply increasing training volume will not further improve performance (Hickson et al., 1981; Hardman et al., 1986; Londeree, 1997; Lucia et al., 2000; Laursen & Jenkins, 2002). In order to further improve changes in gene expression, biochemistry and physiology, a different training stimulus is required.

2.3.2 Endurance training for performance and competition

Although the underlying physiological adaptations associated with improved endurance performance with training are well established, debate exists regarding what is the most efficient training approach in order to induce these adaptations and translate them to performance gains. Reasonably, a combination of training duration, intensity and frequency are the means to attain these gains in performance. However, the intensity of training and how the "day-to-day" training intensity should be distributed is a key issue of debate. In order to quantify intensity, training zones have been created, using ventilatory thresholds and their associated heart rate (HR) values (Lucia et al., 2000; Esteve-Lanao et al., 2005; Esteve-Lanao et al., 2007). Zone 1 is low intensity exercise performance below the first ventilatory threshold (VT1); Zone 2 is moderately high-intensity exercise in an intensity range between VT1 and respiratory compensation threshold (RCT) or second ventilatory threshold (VT2); and Zone 3 is high-intensity exercise performed above RCT (Lucia et al., 2000; Esteve-Lanao et al., 2005; Esteve-Lanao et al., 2007). Interestingly, the intensity characteristics of a professional multi-stage cycling race have been recorded (Fernandez-Garcia et al., 2000; Lucia et al., 2003a; Lucia et al., 2003b). Multi-stage cycling races demand athletes endure a long-duration and high intensity pace throughout the event (Faria et al., 2005a).

Furthermore, during Vuelta a Espana and Tour de France events, cyclists would spend ~75-79 minutes per day doing intense aerobic work (zone 3), and ~97-89 minutes per day doing moderate aerobic work (zone 2) (Fernandez-Garcia et al., 2000). During Vuelta a Espana and Tour de France, 93 minutes per day were spent on flat stages and 123 minutes per day on mountain stages (Fernandez-Garcia et al., 2000). In total, the cyclists were working at an

intensity >70% of VO_2max, where 18-27 of the latter minutes were spent at an intensity >90% of VO_2max (Fernandez-Garcia et al., 2000). In Addition, a professional winner of the short road-cycling time trial during a Tour de France event sustained 70 minutes at an intensity >90% of VO_2max (Faria et al., 2005b). In order to succeed in such world-recognised events, it is clear that cyclists must have or be trained to have certain physiological, biochemical and psychological characteristics. Successful cyclists possess high VO_2max values of ~74 $mL \cdot kg^{-1} \cdot min^{-1}$, the capacity to perform at high workloads (~90% of VO_2max) for longer periods of time, lower circulating lactate and a considerable reliance on fat metabolism even at high power outputs, favourable neuromuscular adaptations, high stroke volume and increased blood volume (Coyle, 1999; Lucia et al., 2000; Lucia et al., 2003b; Faria et al., 2005a; Joyner & Coyle, 2008).

Fascinatingly, these characteristics are supported by genetic studies associating phenotypic features of endurance trained individuals with gene expression analysis (Lippi et al., 2010; Stepto et al., 2009). Furthermore, expression of these genes as a response to endurance training, as well as a chronic alteration in endurance athletes has also been found (Lippi et al., 2010; Stepto et al., 2009). However, in order to gain the mentioned performance changes, endurance athletes have adapted a specific training pattern. Cyclists, runners, cross-country runners, and rowers all share the fact that they perform ~75% of their training at intensities below lactate threshold (zone 1) despite competing at much higher intensities (Lucia et al., 2000; Billat, 2001; Fiskerstrand & Seiler, 2004; Esteve-Lanao et al., 2005; Esteve-Lanao et al., 2007). Table 1 and 2 below support this statement with an example from a longitudinal study showing thirteen professional road cyclists and their training volume and intensity during three training periods; rest, pre-competition and competition (Lucia et al., 2000).

Table 1 - Training volume of the subjects during each of the three periods

	Rest	**Pre-competition**	**Competition**
Average weekly training (km)	267 ± 30	713 ± 28	810 ± 15
Accumulated training volume (km)	500 ± 100	7,433 ± 491	12,767 ± 1027
Data is presented as mean ±SEM.			

(Lucia et al., 2000)

Table 2 - Training intensity of the subjects during each of the three periods

	Rest	Pre-competition	Competition
Low intensity (%)	87.8 ± 3.0	77.8 ± 1.4	76.8 ± 0.4
Moderate intensity (%)	10.7 ± 2.3	17.3 ± 1.2	15.1 ± 1.5
High intensity (%)	1.5 ± 1.0	4.9 ± 0.6	8.1 ± 1.6
Data is presented as mean ±SEM.			

(Lucia et al., 2000)

It has been reported that endurance athletes require a relatively small percentage of their training intensities to be in zone 2 and zone 3 to achieve top performance (Esteve-Lanao et al., 2007). However, challenging the latter statement is a meta-analysis by Londeree (1997) who showed that training at intensities at or near the lactate or ventilatory threshold would improve the threshold for sedentary subjects, however higher intensities may be necessary for trained individuals. Otherwise, detraining may be a consequence with a reduction in lactate and ventilatory thresholds in trained individuals (Londeree, 1997). In support of the latter statements, endurance training will only increase VO_2max, capillary density, oxidative enzyme activity and plasma volume in previously untrained individuals (Billat, 2001; Laursen & Jenkins, 2002; Gibala et al., 2006; Seiler & Tønnessen, 2009; Laursen, 2010; Laursen, 2012). However, in already trained individuals these changes have already occurred and will not further improve by increasing the volume of submaximal training (Billat, 2001; Laursen & Jenkins, 2002; Gibala et al., 2006; Seiler & Tønnessen, 2009; Laursen, 2010; Laursen, 2012). In fact, once the athlete has reached a VO_2max of >60 $mL \cdot kg^{-1} \cdot min^{-1}$, increasing the submaximal (continuous) training volume will not further improve endurance performance (Laursen & Jenkins, 2002).

2.4 Assessment of performance

Comprehensive assessment of performance is more than simply running on a treadmill or riding on a cycle ergometer. There are simple measures that can be used to evaluate endurance, peak heart rate, blood pressure, and the presence or absence of dysrhythmias during exercise. With the use of gas analysers and computers, information about ventilation, oxygen supply and use, metabolism at the muscle level, peak power, and anaerobic capacity

can be assessed noninvasively. With oxygen being the primordial molecule for oxidative phosphorylation, the evaluation of its consumption is important for endurance performance.

2.4.1 Oxygen consumption (VO_2)

Maximum oxygen consumption – VO_2max, is defined as the maximal rate at which O_2 can be taken up and used by the body during exercise at sea level and is a functional quantification of the limits of the cardio-vascular system (Mitchell & Blomqvist, 1971; Barbeau et al., 1993; Washington, 1999; Bassett & Howley, 2000; Bisi et al., 2011). VO_2max has been the most common measurement made in human performance as it provides an index of cardiopulmonary fitness and aerobic capacity which are important features of endurance performance (Mitchell & Blomqvist, 1971; Barbeau et al., 1993). A VO_2max test is used in order to characterise subjects in research, when expressing or prescribing exercise intensity or training (using %VO_2max) and to monitor the effectiveness of endurance training programs (Mitchell & Blomqvist, 1971; Barbeau et al., 1993; Bassett & Howley, 2000). However, the term "maximal oxygen uptake" was characterised and defined by Hill and Herbst in the 1920s. Hill and Lupton's VO_2max model, hypothesised that; 1, there is an upper limit to oxygen uptake; 2, there are individual differences in VO_2max; 3, a high VO_2max is a prerequisite for success in middle and long distance performance; and 4, VO_2max is limited by the ability of the cardiorespiratory system to transport O_2 to the muscles (Hill & Lupton, 1923). Furthermore, in a study, Hill and Lupton (1923) investigated the attainment of a "steady state" for oxygen uptake over time and at different speeds. In these studies, they demonstrated the change in VO_2 over time and how speed influenced the attainment of a "steady state". It was made clear that with higher speed, the VO_2 climb was steeper and time to reach "steady state" was longer (Hill & Lupton, 1923). Furthermore, they confirmed that VO_2 reaches a maximum, beyond which further increases in speed result in no additional increase in VO_2 (Figure 2) (Hill & Lupton, 1923). These findings were supported later by Astrand & Saltin, (1961) who stated that VO_2 increases with higher work rate, however the upper limits of VO_2 with increased speed is about the same (Astrand & Saltin, 1961). Although not described by Hill and Lupton, this "upper limits" and apparent "steady state" has in later literature been accepted as the VO_2 plateau (Bassett & Howley, 2000). However, the claims that Hill and colleagues had established a plateau was strongly criticised by Noakes (1997, 2008). In fact, Noakes amongst others, asserted that the

research of Hill and co-workers "failed to establish the existence of the plateau phenomenon during exercise" (Noakes, 1997; Hale, 2008). The dispute seems to be about the question of a "true" VO_2max, whether it has been achieved and, as the majority of maximal exercise tests are terminated in the absence of the plateau, what is the cause of exercise termination and can this still be called the "true" VO_2max (Bassett & Howley, 2000; Hale, 2008; Noakes, 2008). However, as clear as the concept of a plateau might be, it is common for subjects to complete a graded exercise test to volitional fatigue without achieving a plateau in VO_2 (Howley et al., 1995; Hale, 2008). Therefore, a variety of criteria have been established and used by scientists to characterise a subjects VO_2max: 1. Achieving a plateau in oxygen uptake despite an increase in the external work (< 0.15 $L \cdot min^{-1}$ or < 2 $mL \cdot kg^{-1} min^{-1}$). This is the primary criterion. 2. Respiratory exchange ratio (RER) >1.15, however these values show a large variability among subjects. 3. Heart rate within 10 beats/min of age-predicted value that is found through the calculation, 220-age. 4. Post-exercise venous lactate of more than 8 mM, these values also have a great variability among subjects. 5. Finally, a rating of perceived exertion of more than 17 (6-20 Borg scale) (Brooks et al., 2004; Howley et al., 1995; Hale, 2008).

2.4.2 VO_2max limitations

When discussing the limiting factors for VO_2max in the field of exercise physiology in this thesis, it is with reference to human subjects, without metabolic disease, undergoing maximal exercise tests at sea level. The ongoing discussion as to limiting factors for VO_2max continues, as there are multiple aspects to consider. The potential impairment of VO_2max performance involves four factors: 1, the pulmonary diffusing capacity 2, maximal cardiac output 3, oxygen carrying capacity of the blood and 4, skeletal muscle characteristics (Bassett & Howley, 2000). The pulmonary system can limit VO_2max depending on the health and fitness status of the individual. Due to a significantly higher cardiac output in trained individuals than untrained individuals, trained individuals are more likely to undergo arterial O_2 desaturation during maximal training (Dempsey et al., 1984; Dempsey & Wagner, 1999; Rice et al., 1999).

A higher cardiac output leads to a decreased time for transit of the red blood cells in the pulmonary capillaries. As a consequence, the blood will exit the pulmonary capillaries

before adequately saturated with O_2 (Dempsey et al., 1984; Dempsey & Wagner, 1999; Rice et al., 1999; Bassett & Howley, 2000). Subsequently, the PO_2 (Pa_{O2}) will decrease and there will be a widening of the alveolar-arterial O_2 tension difference (Rice et al., 1999). As mentioned previously, cardiac output is higher in trained versus untrained individuals. In fact, 70-80% of the limitation in VO_2max is linked to maximal cardiac output (Bassett & Howley, 2000). Altering the oxygen carrying capacity of the blood through increasing a person's haemoglobin (Hb) content by removal, storage and subsequent reinfusion has shown to increase VO_2max by 4-9% (Gledhill, 1982; Gledhill, 1985; Bassett & Howley, 2000). Furthermore, skeletal muscle characteristics have also been marked as a limiting factor of VO_2max (Bassett & Howley, 2000). There are multiple factors that induce local muscle fatigue, such as failure of sarcoplasmic reticulum calcium release, impaired sodium/potassium pump activity and slowed cross-bridge cycling due to a variety of metabolic mediators including reactive oxygen species (Hargreaves et al., 1998; Hargreaves, 2000; Reid, 2001; Allen et al., 2008; Amann & Calbet, 2008; Fitts, 2008; McKenna & Hargreaves, 2000). However, during circumstances such as severe acute hypoxia, central fatigue may be quite conspicuous and cause exercise effort to be compromised even before peripheral fatigue develops (Amann & Calbet, 2008).

2.4.3 VO_2max protocols

Maximal rate of oxygen consumption (VO_2max), dates back to the work of Hill and Lupton in 1923 and has been the most commonly measured parameter in applied physiological sciences (Hill & Lupton, 1923; Yoon et al., 2007). VO_2max testing methods used for research purposes during the pre-World War II era were typically intermittent exercise protocols distributed over several days (Hill & Lupton, 1923; Taylor, 1955; Yoon et al., 2007). This was caused by the time consuming process of chemically analysing the gas content of expired air as well as the inadequate control of the exercise equipment (Taylor, 1955; Mitchell & Blomqvist, 1971; Pollock et al., 1976). However, once continuous exercise testing was introduced, validation of several VO_2max testing protocols were undertaken (Yoon et al., 2007). One study assessed three different protocols; the Bruce, Balke and Taylor protocols (see Figure 7), where the stage duration and increment, as well as total test duration varied (Froelicher et al., 1974). It was found that the discontinuous protocol (Taylor protocol) showed significantly higher VO_2max values compared to the two other protocols (Froelicher

et al., 1974). Additionally, investigated in a separate paper was the difference in four different exercise testing protocols; the Balke, Bruce, Ellestad and the modified Astrand on 55 subjects (Pollock et al., 1976), where the only difference found was the VO_2max value between the Astrand and Balke protocols (Pollock et al., 1976). Apart from the aforementioned studies, several others have attempted to assess assorted protocols (Davis et al., 1982; Fairshter et al., 1983; Kang et al., 2001). However, it was not until the study of Buchfuhrer et al. (1983) that the question of what the optimal protocol to use in order to obtain a correct VO_2max was addressed. This study used a variety of exercise tests with 1-minute increments to volitional fatigue on a cycle or treadmill ergometer. The findings were that the test with a duration of 8 – 17 minutes achieved higher VO_2max values than if the protocol duration was less than 8 minutes or exceeded 17 minutes (Buchfuhrer et al., 1983). Actually, these researchers recommended that a VO_2max exercise test protocol last 10 ± 2 minutes (Buchfuhrer et al., 1983). In contrast, it has recently been publicised that exercise test protocols of a duration of less than 8 minutes would result in higher VO_2max values than if the test would last 14 minutes (McCole et al., 2001; Lepretre et al., 2004; Astorino et al., 2005).

Figure 7 - Format of treadmill protocols

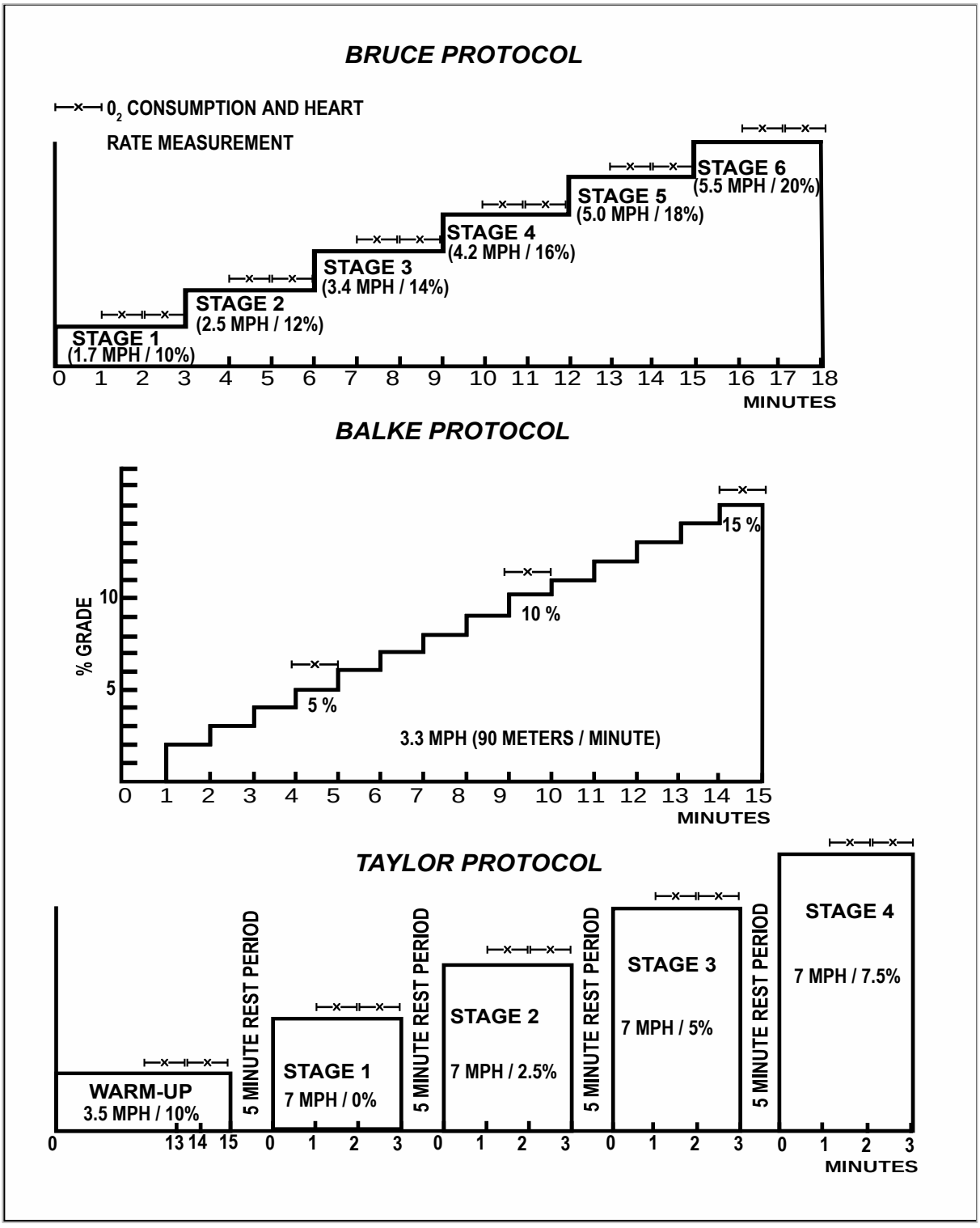


Fig7_The format of three treadmill protocols; The Bruce protocol, Balke protocol and Taylor protocol (Froelicher et al., 1974). The Bruce protocol increases speed and %grade every 3 minutes. The Balke protocol increases %grade every minute, keeping speed consistent throughout. The Taylor protocol, after warm-up, uses 3-minute stages with 5 minutes rest between stages. Each 3-minute stage increasing %grade, keeping speed consistent.

2.4.4 Ventilation thresholds

When an individual performs work in an incremental manner until symptom limitation or volitional fatigue, it is usually said that they are doing progressive exercise protocols (Cross et al., 2012). These types of protocols are frequently used in order to determine parameters such as VO_2, heart rate, maximum work rate or aerobic power (Cross et al., 2012). This type of exercise protocol induces non-linear changes or "thresholds' in gas-exchange and ventilatory parameters prior to exhaustion (Cross et al., 2012). Furthermore, ventilation thresholds 1 and 2, which can be measured during an incremental test, are actively used in research to differentiate between exercise-intensity domains when designing training programs for athletes and in order to provide prognostic values for a clinical population (Yoshida et al., 1997; Carter et al., 2000; Carter et al., 2006; Gitt et al., 2002; Wasserman et al., 2005; Chase et al., 2010; Cross et al., 2010). The ventilation 1 and ventilation 2 thresholds are characterised by inconsistent increases in expired ventilation (V_E) measured by the minute in relation to oxygen consumed and carbon dioxide produced during an incremental exercise test (Beaver et al., 1986; Wasserman et al., 2005). One might expect to observe consequent changes in these parameters as an individual goes through the gas-exchange thresholds during progressive exercise, particularly given that V_E represents the product of respiratory frequency and tidal volume (Cross et al., 2012). Actually, these particular changes in breathing patterns provide useful non-invasive estimates of the gas-exchange thresholds (Cross et al., 2012).

There are two separate and distinct accelerations in respiration frequency during an incremental exercise test that has been observed by other investigators (James et al., 1989; Cheng et al., 1992; Jones & Doust, 1998; Nabetani et al., 2002; Carey et al., 2005; Neder & Stein, 2006; Cannon et al., 2009). The majority of the available evidence proposes that these inconsistent increases in respiratory frequency occur at the gas-exchange thresholds, VT1 and VT2. The practical applications of threshold detection include the prescription of appropriate exercise intensity (Yoshida et al., 1982; Dwyer & Bybee, 1983), measurement of endurance training effects (Poole & Gaesser, 1985; Gaesser & Poole, 1986), and the prediction of endurance performance success (Farrell et al., 1993; Yoshida et al., 1982). In order to determine the appropriate thresholds using ventilatory measurements, several non-invasive analysis techniques have been used. Due to subjective bias inherent in the

visual detection method, a computerised recognition process has been developed in recent years (Orr et al., 1982; Beaver et al., 1986). A moving average filter to smooth breath-by-breath fluctuations in the gas exchange data, V-slope method was created by Beaver et al. (1986). The method entailed using a computerized linear regression analysis of the slopes of carbon dioxide output plotted as a function of oxygen uptake in order to detect the anaerobic threshold (Schneider et al., 1993). In theory, the use of this method allows for a better understanding of pH buffering capacities, for example following an increase in lactate concentrations. Later, the V-slope method was modified by Sue and colleagues (1988) (Sue et al., 1988; Schneider et al., 1993).

When modified, VCO_2 was plotted against VO_2 and a visual line, parallel to the line of identity, was drawn through the data points of the incremental exercise recordings (Schneider et al., 1993). Brought in from the right, in order to facilitate the process, was a 45^0 triangle parallel to the VCO_2 vs. VO_2 (Dickstein et al., 1990; Schneider et al., 1993). The point at which VCO_2 starts to increase more rapidly than VO_2 is then visually identified as the gas exchange threshold (Schneider et al., 1993). However, as several laboratories do not have the equipment to measure breath-by-breath determination of the gas exchange, investigators have further modified the V-slope method (Walsh & Davis, 1990; Zhang et al., 1991; Schneider et al., 1993). It has been found that, by using 30-s sampling periods and applying the modified V-slope method, there is no difference between the gas exchange threshold and the blood lactate threshold (Schneider et al., 1993). For this reason, the V-slope method using the 30-s sampling technique will provide good estimates of the blood lactate threshold (Schneider et al., 1993), a threshold that reflects the anaerobic capacity and limitations in endurance performance.

2.4.5 Endurance capacity test (ECT)

Testing of athletes requires correct identification and assessment of the sport-specific underlying factors. It is well recognised that performance in long-distance events is determined by maximal oxygen uptake (VO_2max), be it the energy cost of exercise and the maximal fractional utilisation of VO_2max in any realised performance or as a set percentage of VO_2max that can be endured as long as possible. This later ability is defined as endurance, and more precisely aerobic endurance, as VO_2max sets an upper limit for the aerobic

pathway. Although a measure of VO_2max has become routine in the physiological testing of elite athletes (Gore, 2000), the correlation between VO_2max and performance may require additional factors with important roles in endurance performance (Conley & Krahenbuhl, 1980; Sjödin & Svedenhag, 1985). Actually, an athlete with a lower VO_2max, compared to another, can achieve a similar oxygen uptake (VO_2; $mL \cdot kg^{-1} \cdot min^{-1}$) during a race by using a higher percentage of VO_2max (%VO_2) (Costill et al., 1973; Sjödin & Svedenhag, 1985). Therefore, it is recognised that additional parameters are important to use when predicting long-distance performance, namely the energy cost of exercise and the ability to sustain a high %VO_2max throughout the entire effort for a given duration (Péronnet & Thibault, 1989; Bosquet et al., 2002). However, the physiological basis for aerobic endurance is subject to ongoing debate. Greater aerobic endurance capacity may be associated with several factors, such as a high percentage of type I muscle fibres, the capacity to store large amounts of muscle and liver glycogen, the capacity to spare carbohydrate by using more fatty acids as energy substrates and the capacity to efficiently disperse heat (Foster et al., 1978; Bosquet et al., 2002).

There are basically three types of tests that can be used in order to assess aerobic endurance based on one single bout at a set intensity: constant-work (CWT), constant-duration (CDT) and constant-power (CPT) (Coyle et al., 1991; Hopkins et al., 2001; Bosquet et al., 2002). During the CWT, a set amount of work or distance is completed as quickly as possible (Hopkins et al., 2001; Bosquet et al., 2002). Similarly, during the CDT, as much work or distance as possible must be covered in a set time (Hopkins et al., 2001; Bosquet et al., 2002). In contrast to the two latter methods, CPT consists of maintaining a constant power output until volitional fatigue, which is defined as the inability to maintain cadence, speed or power (Hopkins et al., 2001; Bosquet et al., 2002), measured in time to exhaustion (Monod & Scherrer, 1965). Moreover, the assessments of CWT, CDT, and CPT correlate well with performance, which has resulted in their frequent use by researchers (Coyle et al., 1988; Bosquet et al., 2002). Coyle and colleagues (1988), conducted a study on a group of cyclists where they demonstrated that an aerobic endurance test performed at 88% of VO_2max was significantly related to the athletes' muscle capillary density and their lactate threshold (LT) ($r = 0.96$, $p < 0.05$). Furthermore, this study showed that the athletes with a longer time to fatigue in the CPT possessed a greater percentage of type I muscle fibres

(Coyle et al., 1988). These findings were further confirmed in 1991, using the CWT and CDT (Coyle et al., 1991). While positive association have been found between CPT, CWT, CDT and aerobic endurance, optimal measurement methodology must be ensured. When performing a CPT in order to assess aerobic endurance capacity, the power output should be established through a $\%VO_2max$ rather than a set absolute intensity (km/h, W or $mL \cdot min^{-1} \cdot kg^{-1}$). If this is not undertaken, it is impossible to separate aerobic endurance capacity from other factors of performance (Bosquet et al., 2002).

2.4.6 Wingate – The peak and average power test

The Wingate Anaerobic Test (WAnT) is a maximal intensity cycle ergometer test of 30 s duration. This test was developed during the 1970s and serves to evaluate anaerobic performance (Beneke et al., 2002). The WAnT is used world-wide and is considered to be the most valid test of anaerobic muscle performance (Bar-Or, 1987). The test is based on the subject cycling at their maximal speed for 30 seconds against a pre-determined braking force. This force is kept constant throughout the test, however the velocity cannot be maintained for more than a few seconds before the subject begins to fatigue and slow down. The mechanical power during a WAnT is measured using the equation: **Power = force x velocity** (Bar-Or, 1987). Force equals the braking force (resistance) that acts on the perimeter of the ergometer like the Lode bike flywheel. The velocity is the distance travelled over time at the flywheel perimeter. Because the crank length is constant, the braking force (Torque in Nm) remains constant throughout the test, and effectively the peddling speed is measured to calculate the power. The work is then calculated by: **Work = power x time** ($P(w) = F(N) \times rev \cdot s^{-1} \times 6\ m \cdot rev^{-1}$). Furthermore, the mean anaerobic power is a function of total work done divided by the time over which that work was completed (Bar-Or, 1987) .

However, the results of the WAnT test depend upon the workload selected for each subject. If the workload is too low the subject will be able to maintain a high level of cadence during the entire test. The Wingate test, which is derived from Cumming's test (1974) (Vandewalle et al., 1987), is the most frequently used anaerobic test and has been studied by many authors since its first description. The same protocols have been adapted to isokinetic cycle ergometers (Vandewalle et al., 1987). The Wingate test involves cycling at maximal speed

for 30 seconds against a resistance related to body weight (40g/kg bodyweight with a Fleisch ergometer; 75g/kg bodyweight with a Monark ergometer) (Vandewalle et al., 1987). The assessment of optimal resistance is difficult because the relationship between force and mean power is parabolic. The value of resistance is either standard (5.5kg with a Monark cycle-ergometer) or related to bodyweight (Vandewalle et al., 1987). In the latter case, a research group has produced different optimal resistances (0.075 kg/kg bodyweight [Bar-Or, 1987]; 0.087 kg/kg bodyweight [Dotan & Bar-Or, 1983]). The low value of optimal resistance which has been proposed by Ayalon et al. (1974) on the Wingate test is probably explained by the fact that the subjects used in their first study were children (Vandewalle et al., 1987).

To allow comparisons of WAnT between subjects, this study has used the suggested constants defined by Bar-Or (1987) (see Table 3).

Table 3 – Constants for comparisons of WAnT between subjects

T (torque) = G (body weight) x c (standard).

Leg ergometry	**X body weight = Torque (in Nm).**
Male adult	0.7
Athlete male adult	**0.8**
Female adult	0.67
Athlete female adult	0.77
Boys (age 7-14)	0.55
Girls (age 7-14)	0.53

(Bar-Or, 1987)

During the WAnT the performance measures are peak power, mean power and the fatigue index (Hopkins et al., 2001). Peak power is the highest mechanical power elicited from the test, taken as the average power over any 5 s period, and corresponds to maximal anaerobic power (Hopkins et al., 2001). Mean power is the average power maintained throughout the six 5 s segments that is supposed to be an index of anaerobic capacity. Finally, the fatigue index is the rate of decline in power during the test expressed as a percentage of peak power (Beneke et al., 2002). Furthermore, the fatigue index reflects the individual's ability

to maintain power over the duration of the test. Individuals are unable to maintain power output throughout the test due to the onset of neuromuscular fatigue (Bogdanis et al., 1996; Beneke et al., 2002).

The 30 s WAnT is frequently used with the aim to compare anaerobic power (maximal rate of anaerobic ATP production) between genders, generations, athletes and various clinical populations (Franklin et al., 2007; Minahan et al., 2007; Franklin et al., 2008). Several short all-out cycling tests have been developed in order to obtain optimal peak power and anaerobic capacity information. Such protocols vary in length, ranging from 20 to 120 seconds (Vandewalle et al., 1987; Laurent Jr et al., 2007). In principle, these "all out" cycling tests are similar to the Wingate test, but their characteristics (e.g. reliability, validity, optimization of braking force) have not been studied as extensively as those of the Wingate test. There is no "gold standard" anaerobic performance test by which to compare the scores of the WAnT to in order to have physiological validity, however the performers with the highest lactate values after the WAnT would be expected to have the highest glycolytic or anaerobic capacities. A validation study by Tamayo et al. (1984) found only a moderate relationship ($r = 0.55$ and 0.60) between mean anaerobic power and blood lactate concentration. However, the relationship between mean anaerobic power and maximal oxygen debt was lower (Tamayo et al., 1984). Furthermore, another research group, using accumulated oxygen deficit as a measure of anaerobic energy release, suggests that the WAnT may not be an appropriate test to use when assessing anaerobic capacity, as it does not exhaust anaerobic capacity (Medbø & Tabata, 1993; Minahan et al., 2007). In contrast, other investigators (Jacobs et al., 1987) supported the test's physiological validity based on findings showing a significant relationship between both the peak anaerobic power and the total work versus percentage and area of fast-twitch fibres. Additionally, the WAnT's validity is supported by the relationship found between peak power and 50 m run time ($r = -0.91$) and vertical jump (Margaria et al., 1969; Jacobs et al., 1987).

The WAnT reliability coefficients (test-retest comparisons) for maximal anaerobic power/capacity are high, ranging from 0.95 to 0.98 (Inbar et al., 1976; Evans & Quinney, 1981). The re-test reliability of the fatigue index can range from values 0.43 to 0.73

(Vandewalle et al., 1987), while the interclass correlation coefficient for repeat WAnT within one week from each other was r=0.98 for mean aerobic power (Evans & Quinney, 1981).

2.5 Training monitoring

Training monitoring is an important task with a continually growing potential. In order for endurance athletes to achieve their fundamental aim, which is to improve their ability to physically perform at a high level for a prolonged pre-determined period of time, the athletes themselves or their coaches must master optimal training monitoring. Elements involved in training monitoring include; psychological, physiological, biochemical and potentially molecular factors such as gene expression (Coutts et al., 2007). Failing to track the changes in these factors may lead to overtraining, staleness and decreased performance in the athlete (Coutts et al., 2007). To achieve selected goals, it is essential to plan efficiently and monitor responses (Coutts et al., 2007). The planning process is a methodical, scientific procedure to help any athlete achieve high levels of training and performance. An organised, systematic training program eliminates the random, aimless approaches still used in some sports today. A well-structured plan provides guidance, direction and scope to a sporting regime. Furthermore, by using relevant monitoring tools, information as to how the body responds and tolerates the applied stress can be recorded and in turn show the efficiency of the training plan (Coutts et al., 2007). This method reveals if the training structure stimulates optimal performance needs (Coutts et al., 2007). If not, there will be an opportunity to manipulate the training plan to better suit individual needs.

Training for a sport or a specific goal can be subject to an emotional experience, capable of influencing both athletes and average recreationally trained individuals. An achievement in sport can create feelings of happiness, while a defeat may result in disappointment and despair. Often the emotional state of an athlete can negatively influence performance during training and competition (Jones, 2003; Coutts et al., 2007; Kellmann, 2010). Emotions in sport were first formulated in 1980 (Jones, 2003): "*a reaction to a stimulus event (either actual or immagined). It involves change in viscera and musculature of the person, is experienced subjectively in characteristic ways, is expressed through such means as facial*

changes and action tendencies, and may mediate and energize subsequent behaviors" (Jones, 2003). An athlete's emotional state can influence performance through motivation, physiology and cognitive functioning (Fry et al., 1994; Jones, 2003; Coutts et al., 2007; Sharp & Masterson, 2007; Kreher & Schwartz, 2012). Furthermore, when failing to optimally monitor an athletic training program, factors such as severe fatigue, immune system deficits, mood disturbance, physical complaints, sleep difficulties and reduced appetite may affect the athlete. When these factors present themselves, it is clear that training monitoring has been absent or failed and performance, as well as the athlete, has been compomised (Fry et al., 1994; Jones, 2003; Coutts et al., 2007; Sharp & Masterson, 2007). Additionally, none of the aforementioned factors may present themselves, only a decrease in performance outcomes. Equally, this is a sign of a failed monitoring program as optimal training stimulus has not been applied in order for the athlete to achieve performance gains. Fortunately, there are several tools that can be implemented in order to prevent or minimise any of the aformentioned situations.

It has already been established in the literature some time ago that heart rate is linearly related to VO_2 during various intensities (Achten & Jeukendrup, 2003). Due to these findings, heart rate values can be used to reveal the intensity of the exercise being performed (Astrand & Rodahl, 1986; Achten & Jeukendrup, 2003; Jeukendrup et al., 1992). In fact, heart rate measurements have become the most commonly used method when intending to monitor intensity of training programs (Achten & Jeukendrup, 2003). This is due to its easy access and immediate use, as well as the stable pattern shown during exercise (Astrand & Rodahl, 1986; Achten & Jeukendrup, 2003). However, as heart rate has an unpredicted variability between subjects, it is more optimal as a monitoring tool when related to other direct indicators of performance capacities such as maximum oxygen consumption (Astrand & Rodahl, 1986; Achten & Jeukendrup, 2003). Furthermore, it has been suggested in earlier studies that heart rate can reflect an over-reached state for an athlete by showing an increase at rest (Dressendorfer et al., 1985; Kindermann, 1986). Over-reaching is a state where the athlete is experiencing fatigue and a reduction in performance due to a disproportionate balance between training and recovery (Halson & Jeukendrup, 2004). However, other studies investigating resting heart rate in athletes in normal and over-reached state could not confirm this statement (Snyder et al., 1993; Halson et al., 2002;

Urhausen & Kindermann, 2002; Jeukendrup et al., 2008). Interestingly, it has been found and confirmed in additional studies that an increased heart rate does appear in a sleeping state in over-reached athletes (Jeukendrup & Van Diemen, 1998; Urhausen & Kindermann, 2002; Achten & Jeukendrup, 2003; Jeukendrup et al., 1992). Additionally, a rating of perceived exertion (RPE) is another tool that can be supplementary to the athlete or coach when assessing the stress (i.e. duration and intensity) put on the body (Kenttä & Hassmén, 1998; Urhausen & Kindermann, 2002). This is performed by the athlete rating the session and/or exercise by how stressful it felt. Because of this, RPE is also a valuable tool in detecting overtraining.

VO_2max has been the most used measurement in human performance assessment as it provides an index of cardiopulmonary fitness and aerobic capacity. Aerobic capacity is the amount of oxygen consumed by the body during exercises in a given time frame. For this reason, VO_2max is related to endurance performance (Mitchell & Blomqvist, 1971; Barbeau et al., 1993). A VO_2max test is used in order to characterise subjects in research, when expressing or prescribing exercise intensity or training (using %VO_2max) and to monitor effectiveness of endurance training programs by assessing changes in oxygen consumption (Mitchell & Blomqvist, 1971; Barbeau et al., 1993; Bassett & Howley, 2000).

During training, metabolic pathways are recruited and involve a large panel of enzymatic reactions to enable the maintenance of physical activity. Such metabolic activity in exercise is associated with cellular gene expression of key proteins. Gene expression in the human white blood cells has been shown to mirror the response to stressors such as training (Büttner et al., 2007). Furthermore, it has been suggested that the level of expression of certain genes is workload dependent (Büttner et al., 2007). Therefore, the future of assessing gene expression signalling mechanisms in response to training has a huge potential in the area of individualized training program design. Already there is evidence that training stressors induce inflammatory reactions of the immune system, where the activation of the pro and anti-inflammatory pathways are dependent upon exercise intensity and duration (Ostrowski et al., 1999; Büttner et al., 2007). With this information alone, the potential to use the immune system as a physiological indicator of a person's ability to recover from stressors induced by various workloads is very real (Büttner et al.,

2007; Nieman et al., 2007). Moreover, the field of predicting gains in performance using molecular classifications is well on its way in the literature (Tremblay et al., 1994; Bouchard et al., 2000; Bouchard & Rankinen, 2001; Liew et al., 2006; Timmons et al., 2010). It is stated that by measuring ~30 gene ribonucleic acid (RNA) expression signatures in the muscle prior to training, VO_2max response to endurance training can be predicted (Timmons et al., 2010). The use of microarray technology allows for the complete investigation of the gene response to an external factor like exercise (Connolly et al., 2004; Fehrenbach, 2007). The future use of microarray technology in exercise physiology might be of special value for monitoring the athletes' training process (Büttner et al., 2007). One prerequisite for this intention would be the identification of robust exercise-induced gene expression profiles or "fingerprints" that can be related to exercise intensity or type (Büttner et al., 2007). However, the available data in the literature show very heterogeneous results, most likely due to different analytical platforms and experimental procedures used (Connolly et al., 2004; Zieker et al., 2005b; Fehrenbach, 2007). Interestingly, less invasive techniques consisting of white blood extraction enable researchers to examine gene expression profiles in leucocytes following exercise stimuli (Büttner et al., 2007). This leucocyte gene expression may provide an understanding of the cellular responses to exercise.

2.6 Endurance capacity and mode of training

It has been accepted in the literature that most of the adaptations that accompany endurance training are the result of an increase in muscle energy demand (Booth et al., 1998). Manipulating volume, duration and intensity of work together with rest durations, can modify oxygen delivery to muscle as well as manipulating metabolic pathways within the muscle cells (Hawley, 2002a; Laursen & Jenkins, 2002). It is these characteristics of a particular training program that creates the adaptation at the molecular and cellular level that ultimately produces changes at the systemic level (Laursen & Jenkins, 2002; Laursen et al., 2005). Throughout the literature it has been shown that an increase in physical work capacity is the result of an increased delivery of oxygen to the working muscles and subsequent increased uptake and utilisation of this oxygen in these muscles (Zavorsky, 2000; Thompson et al., 2001; Laursen & Jenkins, 2002).

In untrained individuals it seems that endurance training induces an increase in oxygen delivery and extraction and an increase in fat metabolism in working muscles (Thompson et al., 2001). As a result of this physical work capacity increases because the muscle contraction becomes more efficient through factors such as; increases in mitochondria size and number, enzyme content and capillary density (Laursen et al., 2002). Conversely, a flaw with endurance training falls within its high volume and low intensity nature. When one adapts to endurance training, the increase in performance is a result of adaptation to this sub-maximal workload (Zavorsky, 2000). Even though it seems that training adaptations are directly related to the volume of training, there obviously is a maximal duration beyond which additional stimulus will not result in further performance increases (Hawley, 2006). This in turn suggests that control mechanisms signalling adaptive responses are ultimately titrated by exercise duration.

Therefore, further improvements in performance will require a different training stimulus outside the parameters of high volume, low intensity, and often continuous exercise (Laursen & Jenkins, 2002). Importantly for the athlete, the above reassures that simply training more will not achieve more in terms of adaptation and performance. Hawley and colleagues (1997) in their paper identified several physiological traits that are positively related to successful performance in endurance type sports. For an endurance athlete wanting to compete at a high level in an endurance event, they need to have 1) a high maximal aerobic power (VO_2max), 2) the ability to sustain a high percentage of VO_2max for continued periods (sustainable power), 3) a high power output or speed at the lactate threshold, 4) the ability to endure high absolute power outputs or speeds and resist the onset of muscular fatigue, 5) an efficient/economic technique and, 6) the ability to utilize fuel during continuous exercise at high work rates (Hawley et al., 1997).

By examining the exact physiological parameters that are identified as highly correlated with endurance performance during endurance events, one might acquire some insight into how high intensity training may benefit endurance performance. The following section is a review of the processes that regulate the variables associated with the delivery of oxygen to working muscles and assists the utilisation of oxygen in the active tissue. In a review, Laursen et al. (2002) stated that there was no change found in HRmax in response to

endurance training, although an increase in stroke volume and left ventricular contractile force was observed. An increase in stroke volume has been suggested to be driven by an increase in cardiac filling pressure, which leads to an increase in end-diastolic volume (Laursen & Jenkins, 2002). In contrast, a review by Zavorsky (2000) described 12 studies that documented a definite decrease in HRmax in response to aerobic exercise. This study suggested a possible alteration in HRmax by 3% - 7%. The authors suggested that alterations to HRmax are caused by extrinsic factors such as; an increase in plasma volume, increase in baroreflex function and intrinsic factors such as; alterations in the SA node and a decrease in beta-adrenergic receptor number and density on cardiac myocytes (Laursen & Jenkins, 2002; Zavorsky, 2000). An increase in plasma volume through training or heat acclimatisation has been reported as important in promoting cardiovascular stability and to improve thermoregulation during prolonged exercise (Back et al., 2000; Zavorsky, 2000; Laursen & Jenkins, 2002; Lacerda et al., 2007).

The mechanisms leading to an increase in endurance capacity following high intensity training that have been examined and suggested to have a positive effect on performance, are an increase in heat tolerance and cutaneous blood flow and/or sweat rate (Laursen & Jenkins, 2002). Also, in a study by Gisolfi (1973), an association between volitional fatigue and elevated core temperature was reported, which supports the suggestion that high intensity training can increase temperature regulation. High intensity training has been shown to increase work heat tolerance in an active population (Laursen & Jenkins, 2002), however this is yet to be investigated in athletes.

Peripheral changes associated with endurance capacity, in contrast to central changes, refer to the working muscles increased capacity to produce and utilise ATP. In part, this change is due to an increased enzymatic capacity of skeletal muscle tissue which will increase the rate of energy production (Ross & Leveritt, 2001). Furthermore, an increased amount of substrate stored in the muscle can increase total energy production (Ross & Leveritt, 2001).

Only a few studies have investigated the essential metabolic adaptations accountable for the increase in endurance performance following high intensity training in trained athletes (Westgarth-Taylor et al., 1997; Creer et al., 2004). In the study of Creer et al. (2004) a

significant increase in motor unit activation, lactate and total work output after 4 weeks of high intensity training was reported. Moreover, Westgarth-Taylor et al. (1997) found a significant increase in 40km time trial, peak power and time to fatigue at peak power performance in their athletes with 3 weeks of high intensity training. Conversely, when Westgarth-Taylor and colleagues investigated the activities of HK, PFK, CS, and 3-hydroxyacyl-COA dehydrogenase in response to this exercise, they did not find a significant change. This might suggest that the enzymes measured by the authors might not have been factors contributing to the increased performance.

During high intensity exercise pH drops and will potentially cause fatigue (Green, 1995; Hargreaves et al., 1998). In a review by Ross & Leveritt (2001) the well-known ability of the muscle to use various buffering mechanisms for offsetting the change in pH was reiterated. These various buffering mechanisms include the chemical buffers bicarbonate, phosphate, proteins and haemoglobin in red blood cells (Ross & Leveritt, 2001). Other studies (Jacobs et al., 1987; MacDougall et al., 1998) have found an increase in PFK enzyme activity (enzyme that catalyses the phosphorylation of the glycolytic intermediate fructose 6-phosphate (Ross & Leveritt, 2001). Even though it has been extensively shown that HIT increases both aerobic and anaerobic capacity, Westgarth-Taylor et al. (1997) showed that there was an unchanged activity in related enzymes. Dawson et al. (1998) reported that even though VO_2max increased after high intensity training, citrate synthase activity decreased. However, in recent studies (Burgomaster et al., 2005, Burgomaster et al., 2006; Gibala, 2006; Burgomaster et al., 2008; Gibala, 2009; Little et al., 2010) a significant increase in citrate synthase activity after low volume high intensity exercise was found. However, these studies were performed in recreationally active subjects, not trained endurance athletes. These findings support the suggestion that elite athletes already possess these characteristics of muscle metabolism.

2.6.1 Interval principle and mechanism of effect

Interval training used in sports is based on short to long, repeated bouts of relatively high intensity exercise which is equal or superior to maximal lactate steady-state velocity (Billat, 2001). Furthermore, between the repeated bouts, there are recovery periods of rest or light exercise (Billat, 2001; Laursen & Jenkins, 2002; Laursen et al., 2002; Laursen et al., 2005).

Through this method, a large amount of work can be obtained at an extremely heavy load, placing a clear submaximal load on the circulatory and respiratory system by applying suitable work and rest periods (Astrand et al., 1960b). Furthermore, the total amount of work can be divided into suitable periods in such a way that training of larger muscle groups can be induced without simultaneously loading the respiratory and circulatory organs (Astrand et al., 1960b). Additionally, by choosing longer work periods such as 2 – 3 minutes, a high training effect of the respiration and circulatory system can also be obtained (Astrand et al., 1960b). During interval training there is a slower accumulation of lactate and in return a delay in the onset of fatigue (Billat, 2001). This is due to the replenishment and subsequent reutilisation of the phosphagen reserves which enable the athletes to accomplish large quantities of work at very high intensities (Billat, 2001). Furthermore, a greater amount of work can be achieved with interval training that also allows for larger peripheral training adaptations. A study performed by Sabapathy et al. (2004) showed that individuals with limited lung function were able to complete a significantly greater total amount of work during interval training (71 ± 32 kJ) than during continuous exercise (31 ± 24 kJ) (Sabapathy et al., 2004).

Interval training has traditionally been used across many sports such as track & field, cross country, and swimming. It is a training method appropriate for a wide range of sports and activities through manipulation of several variables: Interval duration, heart rate, number of repetitions, sets and rest intervals. Interval duration is the distance or time of the exercise interval which is determined by the requirements of the sport or activity the athlete is training for (Billat, 2001). Measuring an athlete's heart rate to determine a percentage of the athlete's HRmax can be used to determine the level of stress experienced by the athlete (Billat, 2001). However in general, shorter/higher intensity intervals are associated with a greater number of repetitions and sets (Billat, 2001). As the work interval is lengthened, the number of sets and reps usually are reduced (Billat, 2001). Rest Interval is the time period between consecutive exercise intervals and the duration of rest or active recovery is determined by how quickly an athlete recovers or the goal of the session.

2.6.2 Interval training

While interval training can be traced as far back as 2000 years (Bourne, 2008), this method of training was developed in more detail circa 1910 (Billat, 2001). Around 1910, the Finn Paavo Nurmi and his coach Lauri Pikhala applied an interval system of training by focusing on alternating fast and slow runs. In some cases they would ramp up the effort, while decreasing the distance (Billat, 2001). For example, 4 – 7 km runs, with fast speed over the last 1 – 2 km, immediately followed by four to five sprints. Alternatively, they would begin with a set of sprints, followed by a longer distance run (e.g. 3km) at 75-90% of maximum effort. By the 1930's, the Swedish coach Gosta Holmer had developed a different style of interval training. His style of training required the athlete to vary the speed based on how they felt. So, during a long run, an athlete may alternate between a fast and a slow pace or between a fast and a medium pace or between a medium and a slow pace. The Swedish word for this type of training is "Fartlek" which directly translates to speed play. Fartlek continues to this day to be a very popular form of training for runners.

German coach Woldemar Gerschler observed the Finns and Swedes and subsequently determined that there were opportunities to include more speed work in interval training programs. His system focused on greater intensity of effort because the periods of rest or light running that followed allowed for partial recovery prior to the next hard effort. Rudolf Harbig's coach, Woldemar Gerschler and his training method resulted in world-record performances (Coyle, 2005). Harbig is mainly remembered for his 400 and 800 metre track world records in 1939. When Harbig was first 'spotted' by Woldemar Gerschler in 1934, he was 20 years old and was a "2:04" 800 runner (Bourne, 2008). However, within three months Gerschler had helped Harbig to improve his 800 m time to 1:58.2 (Bourne, 2008). In 1939 Harbig set a world record in the 800 metres with a time of 1:46:6 and in the same year he set a world record in the 400 metres with a time of 46.0 seconds (Bourne, 2008). This 400 metre record stood from 1939 to 1948 while his 800 metre time remained in the record books from 1939 to 1958 (Bourne, 2008). An example of a training program of Harbig in the lead up to his two records in 1939 is below:

" 7/13/39: Workout recorded prior to 800 metre world record in Milan, Italy on 7/5/39 in a time of 1:46:6. Jog 20 minutes, 600 metres in 1:27 seconds; jog 10 minutes; 300 metres in 39.6 seconds; jog 10 minutes; 500 metres in 66.7 seconds"

"8/8/39: Workout recorded prior to 400 metre world record in Frankfurt, Germany on 8/12/39 in a time of 46.0 seconds; Jog 20 minutes; 3 x 250 metres in 30.6, 30.2 and 29.8 seconds with a 10 minute jog after each run"

(Bourne, 2008)

Finally, in the 1960s scientific studies were conducted on interval training (Billat, 2001). The Swedish physiologist, Per Olaf Astrand and his group examined the immediate and long term effect of interval training on metabolism (Astrand et al., 1960a). The group assessed the same work performed, at the same power output but at different work durations; 0.30, 1, 2 and 3 minutes (Astrand et al., 1960a). The research group found that by splitting the cycling exercise into shorter periods (0.30 and 1 minute) of work and rest, the training only produced a submaximal load of 63% of VO_2max with lactate levels reaching 2 mmol/L (Astrand et al., 1960a). This submaximal load on the circulatory and respiratory system could easily be tolerated for one hour (Astrand et al., 1960a). Whereas the longer work periods (2 and 3 minutes), produced close to or at VO_2max loads with a blood lactate of 16.6 mmol/L, could only be tolerated with extreme strain (Astrand et al., 1960a, b & c). As an explanation to the low levels of blood lactate recorded in response to the short training periods, it was proposed that myoglobin functioned as a storage of oxygen during the short spells of heavy load (Astrand et al., 1960a; Billat, 2001). This was confirmed in another paper by the same authors where they calculated oxygen storage capacity to be 0.43L, representing approximately 10% of the maximal accumulated oxygen deficit acquired during an all-out exercise bout of 2 minutes (Astrand et al., 1960a). Myoglobin represents an oxygen store which is used in the initial work phase before respiration and circulation reaches the values corresponding to the oxygen demand (Astrand et al., 1960b; Billat, 2001). In 1961, Astrand and Saltin demonstrated that after warming up, VO_{2peak} could be attained within 1 minute due to the acceleration of the oxygen kinetics at high work rates (Astrand & Saltin, 1961).

Recently, the application of interval training has been thoroughly reviewed and discussed as the training programs of the world's best runners have been revealed. As an example Wilson Kosgei Kipketer (born 12 December 1972) is a Kenyan born Danish former middle distance runner. He holds the current indoor world records at the 800 and 1000 metre distances. While he dominated the 800 m indoor distance for a decade, remained undefeated for a three-year period and ran eight of the 17 current all-time fastest times, he never won an Olympic gold medal. However, he did win gold medals in three successive editions of the IAAF World Championships in Athletics. Kipketer's 800 m indoor world record stood for almost 13 years. An example of Kipketer's use of intervals in his training program is provided:

Day 1: 3 x 2000m or (2 x 1,200m) + (1 x 800m) + (2 x 400m) 5000m pace
Day 2: Fartlek Run
Day 3: 6 to 8 x 800m 3000m pace
Day 4: Distance Running
Day 5: 16 to 30 x 200m alternating with 10 x 400m 1500m pace
Day 6: Rest day if race the next day, or fartlek if not
Day 7: Race or time trial
Day 8: 4 to 6 x 400m or 9 x 300m 800m pace
Day 9: Distance running on roads
Day 10: 1 x 300m + 2 x 200m + 4 x 100m + 8 x 60m 400m pace
http://members.iinet.net.au/~peterq1/run/aths.html

Studies have shown that interval training with a 1:1 minute work/rest ratio is associated with significantly lower values for oxygen uptake, carbon dioxide output, expired ventilation, heart rate, plasma lactate concentration, and ratings of breathlessness in COPD patients (Sabapathy et al., 2004). Furthermore, studies have also found decreases in cardiovascular-disease risk factors in obese adolescents (Rakobowchuk et al., 2008; Tjønna et al., 2009); increases in physical and cardiac performance following coronary bypass surgery (Meyer et al., 1990) and a reversal of the risk factors of metabolic syndrome (Tjønna et al., 2008) in response to interval training. Another study found that interval training increased proteins that transport fatty acids across the mitochondrial membrane (Talanian et al., 2007).

Additionally, interval training has been shown in several studies to increase skeletal muscle enzyme activity (MacDougall et al., 1998; Gibala et al., 2006; Talanian et al., 2007) and the capacity of muscle to oxidise carbohydrates and fat through certain gene expression pathways (Chilibeck et al., 1998; Burgomaster et al., 2008) to the same extent as traditional continuous aerobic training. Furthermore, while aerobic training improves aerobic fitness, it has been reported that high-intensity, repeated anaerobic efforts improve both anaerobic and aerobic fitness (Tabata et al., 1996). While the energy for muscle contraction during maximal exercise lasting less than 20 seconds is primarily derived from anaerobic metabolism (i.e. the phosphagen system and glycolysis), the contribution of aerobic metabolism increases when short sprints are repeated (see section on energy systems for further details).

During the recovery bout of an interval session, heart rate declines at a proportionally greater rate than the return of blood to the heart resulting in a brief increase in stroke volume (the amount of blood the heart pumps with each beat) (Bogdanis et al., 2007). This increase places an overload on the heart, which strengthens it and enables the skeletal muscles to be cleared of waste metabolites quicker, owing to the elevated rate of blood flow when there is little demand for activity from the tissues (Bogdanis et al., 2007). Since stroke volume peaks during the recovery interval and there are many recovery intervals during an interval workout, stroke volume peaks many times providing a stimulus for improving maximum stroke volume and thus for improving the capacity of the oxygen transport system (Bogdanis et al., 2007). Also, during the recovery intervals a significant portion of the muscular stores of quick energy—adenosine triphosphate (ATP) and creatine phosphate (PC)—that were depleted during the preceding work period are replenished via the aerobic system to again be available as an energy source (Bogdanis et al., 2007). Actually, the literature suggests that implementing HIT protocols that use a high percentage of VO_2max optimally stimulates the oxygen transport and utilization system and therefore provides the most effective method for enhancing VO_2max (Laursen & Jenkins, 2002; Midgley & McNaughton, 2006; Midgley et al., 2006; Buchheit & Laursen, 2013).

Although the evidence as to why training protocols of this intensity are needed seems unclear, it could be argued that intensities at or above VO_2max allow for recruitment of type

II muscle fibres and elicit near to maximal cardiac output (Buchheit & Laursen, 2013; Gollnick et al., 1974). This results in joint signalling of oxidative muscle fibre adaptation and myocardium enlargement (Buchheit & Laursen, 2013). In order to create an optimal adaptation of the cardiovascular and peripheral system, it has been suggested that athletes should spend several minutes per HIT session in their "red zone", which usually means achieving an intensity greater that 90% of VO_2max (Billat, 2001; Laursen & Jenkins, 2002; Midgley et al., 2006; Midgley et al., 2007; Buchheit & Laursen, 2013). Accordingly, there has been a growing interest within the sport science community to identify training protocols the will allow the athletes to maintain an intensity of >90% for the longest possible duration (Buchheit & Laursen, 2013). In contrast however, there is a limited understanding of the regime-response relationship between training loads and induced changes in physiological capacity and performance (Buchheit & Laursen, 2013). Additionally, the studies investigating this generally show a large inter-individual response (Bouchard & Rankinen, 2001; Vollaard et al., 2009).

2.6.3 High intensity and sprint interval training

Interval training is widely used today across the exercise and fitness industry. However, the high intensity interval training (HIT) approach to interval training has become more popular and known in recent years through a publication by Tabata in 1996. Furthermore, HIT program optimisation research in cyclists has shown that repeated sprint interval training (SIT) may be equally as effective as more traditional HIT programs for improving endurance performance (Laursen et al., 2002). An investigation was conducted which assessed 20 seconds of SIT (on an electronically braked cycle ergometer) with intensity set to approximately 170% of VO_2max. The bouts were followed by 10-second rest intervals. The entire session lasted for 4 minutes (8 cycles of 20s of SIT to 10s rest) and was implemented 4 times per week in addition to one day of steady state training for six weeks (Tabata et al., 1996). This was compared to a group that completed training at 70% of VO_2max (at steady state) 5 times per week for six weeks. Their results demonstrated that the SIT group made similar gains in physiological measures when compared with the steady state group. Despite the steady state group achieving a higher final VO_2max, the SIT group recorded lower baseline VO_2max values than the steady state group, and thus produced a higher overall increase in VO_2max values. More profoundly, the SIT group had also achieved significant

gains in their anaerobic capacity (Tabata et al., 1996). This preliminary study showed that high intensity; low volume training could generate large increases in endurance capacity. This type of training is now well known within the sport and exercise industry as the Tabata Method (Tabata et al., 1996) and has many work-rest ratio versions that are utilised in the fitness industry. Furthermore, current highly successful strength and conditioning coaches, such as Michael Boyle, uses HIT and SIT as a cornerstone of their routines (www.strengthcoach.com). In an article by Boyle, he advocated that high intensity interval training (HIT) be performed in 3-4 week duration periods as a maximum, due to the intensity and demand that this type of training places on athletes (www.strengthcoach.com). More recently, a small number of researchers have begun to measure and characterise the physiological, biochemical and gene expression changes induced by SIT (Burgomaster et al., 2005; Burgomaster et al., 2006; Gibala et al., 2006). These research groups devised a laboratory-based model of SIT, frequently using the Wingate anaerobic cycling process (30s) to examine these changes. Despite the preliminary research which has begun to examine the more complex effects of SIT on athletes, significant research is required to further the consequences of this type of stressful training on gene expression levels. Furthermore, research should compare these changes with traditional physiological and performance measures to inform the best clinical application of SIT.

It has been estimated that a 30s Wingate bout has a 16% aerobic contribution, a 56% glycolytic contribution and a 28% ATP-PC contribution (Beneke et al., 2002). During the Wingate process, glycolytic power typically peaks within the first 15s of high power exercise, whereas aerobic metabolism contributes in a delayed manner, yet remains significantly involved (Beneke et al., 2002). In contrast to Beneke et al. (2002), some research (Hawley, 2002b) indicates that oxygen is used from the beginning of a sprint test, suggesting that mitochondrial ATP synthesis was triggered after a surprisingly brief duration. One explanation for this discrepancy between study findings is that the warm-up was sufficient to provide the mitochondrial substrates, ADP and Pi, to activate oxidative phosphorylation by type IIa and type I myocytes. Based on the above two papers, it is clear further research is required to investigate the energy systems involved in sprint tests such as Wingate.

Relatively few studies have examined the physiological and performance responses of a modified training program on already trained athletes. One of the reasons for this might be the difficulty of altering the athlete's training program to accommodate the scientist's strict testing parameters. Currently, sports scientists and exercise scientists make recommendations to athletic coaches based on training studies performed primarily on sedentary and recreationally trained individuals. As highly trained endurance athletes already possess high aerobic capacity, lactate threshold and economy (Laursen & Jenkins 2002), HIT and SIT seems to be the most effective way to achieve improvements in performance.

It must be reiterated that SIT is also able to induce many of the same molecular, biochemical and physiological adaptations that are gained through endurance training. These include the ability to sustain a given sub-maximal workload for a longer period of time or achieve a higher average power output over a fixed distance or time (Gibala et al., 2006). Conversely, it should be noted here that whilst HIT and SIT can be exploited to target different energy systems, sport specific training cannot be forgotten; e.g. a short period of endurance training (5–7 days) has been shown to increase glycogen availability but reduces the rate of glycogen catabolism during matched-work exercise (Green et al., 1991; Gibala et al., 2006), resulting in improved endurance capacity (Green, 1995; Gibala et al., 2006) but diminished anaerobic performance. To reiterate, specific training stimuli can either enhance or decrease performance ability and must therefore be understood thoroughly from a scientific perspective to help select the most appropriate stimulus for individuals. In this regard it is interesting to note that many studies have shown that subjects undertaking more intense work bouts over shorter time periods induce similar adaptations to traditional endurance training. There are studies that have reported increased VO_{2peak} after 14-24 sprint interval-training sessions performed over 2-8 weeks (McKenna et al., 1997; Dawson et al., 1998; MacDougall et al., 1998). Over these weeks the training intervention was performed at maximum intensity and, despite this relatively brief stimulus, the researchers found significant increases in VO_2max, maximum power and in glycolytic and oxidative marker enzymes (McKenna et al., 1997; MacDougall et al., 1998). Such a significant increase in VO_2max and muscle oxidative enzymes would be traditionally unexpected given the

nature of the training intervention, as changes of this magnitude are usually associated with high volume sub maximal training intensity during the week (MacDougall et al., 1998).

The finding of an increase in VO_2max is however somewhat conflicting with later studies that investigated the effect of low volume, high intensity training over a period of 2 weeks and its effect on endurance performance (Burgomaster et al., 2005; Burgomaster et al., 2006; Gibala et al., 2006). Burgomaster and colleagues (2005) and Gibala and associates (2006) used a protocol which had the subjects perform the intervention on a cycle ergometer for 4-7 repetitions of a 30s Wingate protocol with 4 minutes of recovery in between bouts. Burgomaster et al. (2006) reported in two different studies that, with only three session of HIT per week for two weeks, VO_2max remained unchanged. However, they could see a substantial increase in their subjects' time-trial performances. In another study, Gibala et al. (2006) compared the difference between high intensity training and sub maximal endurance training. In this particular study, the SIT group carried out a total work output of ~360 kJ, while the endurance training group performed ~6500 kJ. They found a significant increase in muscle buffering capacity and glycogen content in both the endurance-training group and the high intensity group. These findings indicate that as little as 2.5h training time commitment or ~360kJ total work over two weeks with SIT is a time efficient training strategy to increase skeletal muscle oxidative capacity and induce specific metabolic adaptations that are comparable to traditional endurance training.

The scientific literature concerning interval training has not yet investigated the effects of various regimes of HIT or SIT on endurance capacity. However, examining the existing literature collectively it is evident that most combinations of this type of training intervention have a positive outcome on endurance capacity. Furthermore, it also appears that higher repetition bouts have a greater effect on maximum oxygen carrying capacity, whereas fewer bouts seem to result in an increased economy by lifting anaerobic thresholds. As described earlier, past investigations were primarily performed on sedentary healthy or active and healthy subjects where there are substantial gains to be made in the areas of aerobic capacity, lactate thresholds and basic economy of motion. Laursen at al. (2002) investigated the effect of high intensity training on highly trained cyclists. The researchers found that by incorporating high-intensity interval training twice per week for 4 weeks,

trained cyclists could significantly increase their 40-km time trial performance. In a different study with the same training protocol, Laursen et al. (2002) found significant gains in the subjects' time trial performance, VO_{2peak}, ventilation thresholds and anaerobic capacity. However, the researchers did not find any change in plasma volume. Expansion of plasma volume has been regarded as the most important event responsible for improved physical work-capacity following short-term exercise training in untrained individuals (Laursen & Jenkins, 2002).

However, highly trained cyclists already possess elevated levels of plasma volume, which might explain the lack of increase in this parameter despite enhanced performance (Laursen et al., 2002). With this in mind, Laursen et al. (2002) suggest that adaptations to this type of training are peripheral rather than central. There are few papers which have investigated the neuromuscular response to SIT, however it has been shown that neuromuscular fatigue is protocol dependant (Buchheit & Laursen, 2013). Furthermore, high-work intensities creating metabolic stress at the muscle level, may be enough to induce neural and muscular adjustments (Buchheit & Laursen, 2013). At this stage, it is clear that further research is needed to investigate and elucidate the various biological adaptations in response to HIT and SIT and importantly, a regime-response relationship. Furthermore, this research should occur with a trained group of athletes.

2.6.4 Training delivery/regime

The variability involved in manipulating a "regime" of training required to achieve a particular performance response are combinations of frequency, duration, intensity and type of activity (Kesaniemi et al., 2001; Powers & Howley, 2007). Frequency is used when describing the number of training sessions performed during a time period, such as during a day or a week (Kesaniemi et al., 2001). Examples can be made from athletes such as triathletes, who would often do more than one session a day. They might run in the morning and swim in the afternoon. Furthermore, duration refers to the number of minutes that each training session would last (Kesaniemi et al., 2001), such as a 45 minutes run in the morning and a 60 minutes swim in the afternoon. The estimated effort or energy cost of exercise sessions is described as intensity (Kesaniemi et al., 2001). Athletes can use measures, such as the percentage of maximal heart rate, percentage of VO_2max, rating of

perceived exertion and the lactate threshold to ensure the sought intensity for the session (Powers & Howley, 2007). As an example, 45 minutes run in the morning at 80% of heart rate max and a swim in the afternoon at 60% of heart rate max. Type of activity is also an important factor in designing the appropriate regime of preparation for an athlete, as the need for sport specific training is imperative.

Elite cyclists racing in a pack have been shown to randomly vary their work rates from around 50% to 100% of the peak sustained power output, independent of the cycling track (Powers & Howley, 2007). A study by Stepto et al. (2009) investigated the varying effect of interval training intensity on 40-km time-trial performance in 20 male endurance cyclists. The cyclists performed a 25-kJ sprint test, an incremental test to determine peak aerobic power (PP) and a simulated 40-km time-trial on a cycle ergometer. They were then randomly assigned to one of five types of interval-training session: 12 × 30 s at 175% PP, 12 × 60 s at 100% PP, 12 × 2 min at 90% PP, 8 × 4 min at 85% PP, or 4 × 8 min at 80% PP (Stepto et al., 2009). Cyclists completed 6 sessions over 3 weeks, in addition to their usual aerobic base training. All laboratory tests were then repeated. Performances in the time trial were highly reliable when controlled for training effects (coefficient of variation = 1.1%). The results showed statistically significant (P = 0.005) changes and predicted greatest enhancement for the intervals performed at 85% PP and 175% PP (Stepto et al., 2009). Intervals performed at 100% PP and 80% PP did not produce statistically significant enhancements of performance. Furthermore, frequency, duration, intensity and type of activity combinations have been found to yield reactions at the molecular and gene expression level, which in turn creates a product of adaptation. Globally in the literature, the regimes investigated are 5 s – 60 s of maximal effort sprints with 1 – 18 sessions across 1 – 7 wks, with 1 – 10 repetitions per session and 4 - 60 minutes recovery (Appendix A for further details). Billat (2001) published an interesting table where they identified what training regimes would most likely produce which physiological and competition velocity (see Table 4). According to this table, the regimes recorded in Appendix A would relate to competition distances of 800-5000 metre running with an intensity of 95 – 130 % of VO_2max (Billat, 2001). In contrast to this, recent research has found SIT to positively modify endurance performance with as little as six sessions and between 24 – 42 repetitions over two weeks. Additional to this study's performance changes, changes in pyruvate oxidation

and increased muscle oxidative potential was also found (Burgomaster et al., 2005; Gibala et al., 2006). Moreover, a study by Bailey and colleagues (2009) found, by measuring the same volume of work as the latter studies but compared with an endurance group doing the same total work in KJ, that the SIT group had an enhanced fractional muscle O_2 extraction, faster VO_2 kinetics and an increased tolerance to high-intensity exercise. Furthermore, it has been shown that as little as eighteen repetitions of HIT per week for two weeks reduces metabolic risk factors in a young and sedentary population (Babraj et al., 2009).

Whether as few as eighteen repetitions of SIT over two weeks can improve endurance performance has not yet been investigated. Furthermore, the question of the preferable session frequency also remains.

Table 4 – Different types of interval training and their velocities

Intensity	Physiological and competition velocity	Time limit at this velocity (min)	Time spent at VO_2 max (min)	Maximal blood lactate level (mmol/L)	Aerobic metabolism to energy (%)	Anaerobic interval training	Aerobic interval training
115-130	v1000m;v800m	3-2	2-1	15-18	75-65	-6 x 30 s; R=30 s (rest); -60 s, -45 s, -30 s, -45 s, -60 s; R = 5 min (rest)	-20 x 10 s; R =10 s (rest))
105-115vmiles; v1500m	vmiles; v1500m	6-4	4-2	13-15	85-80	-6 x 1 min; R = 3min (rest); -3 x 500m at v1500m; R = 3min (rest)	-15 x 15 s; R = 15 s at 50% v VO_2max
100-105	v VO_{2max};v3000m	8-6	5-4	11-13	90-85	-3 x 1000m at v3000m; R = 3 min (rest)	-20 x 15 s; R = 15 s at 50% v VO_2max
95-100	v5000m	15-8	10-5	9-11	95-90	-5 x 1000m at v5000m; R = 3 min (rest)	-25 x 15 s; R = 15 s at 50% v VO_{2max}; -6 x 3 min; R = 3 min 50% v VO_2max
90-95	v10 000m and critical velocity	30-15	1-10	7-9	97.0		3 x 3000m at v10 000m; R = 3 min (rest)
85-90	Velocity for the record of the hour	60-30	0	5-7	98.0		-2 x 20 min; R = 3 min at 70% v VO_2max
80-85	Maximal lactate steady state	80-60	0	3-5	99.0		-2 x 30 min; R = 3 min at 70% v VO_2max
75-80	Marathon velocity	150-80	0	3-3.5	99.0		-2 x 15km; R = 1 km at 70% v VO_2max
R = recovery between series (i.e. set of several repetitions); v VO_2max = velocity at maximal oxygen uptake; vxm = average velocity over x metres.							

Classification of the different types of interval training according to the specific velocities of a race, the time limit of these velocities and the 'physiological velocities': the velocity of the maximal oxygen uptake (VO_2max) the critical velocity (i.e. the asymptote of the velocity-time limit relationship), and the velocity at maximal lactate steady state (Billat, 2001).

Frequency, duration, intensity and type of activity combinations yield reactions on the molecular and gene expression level, which in turn creates a product of adaptation. For this reason, it is important to investigate the gene expression responses to different training regimes in order to optimize training monitoring and performance.

2.7 Genes and exercise

Multiple studies investigating gene expression responses to exercise have been informative with data being collected throughout several investigations. The experiments that were carried out were diverse and dependent on the type, intensity, and duration of exercise. In addition, the training statuses of the athletes or participants, as well as environmental conditions, were taken into consideration in some of these studies (Booth et al., 1998). Nevertheless, to date no definitive or complete list of altered expression of genes in response to exercise can be drawn. Less is known on the comprehension of the underlying regulatory mechanisms that take place when exercise is undertaken. Established microarray technology has permitted a higher quality of gene expression analysis since researchers can interrogate both the whole transcriptome as well as temporal snapshots of the actual transcriptome responses to a particular stimulus. This technology has therefore become a widely used tool for comprehensive analysis of gene expression and is applied to investigate responses to diverse stimuli through a possible systemic analysis approach (Kerr & Churchill, 2001; Verducci et al., 2006). This data may help researchers to optimally characterize and define the complex stress responses to acute and chronic types of exercise at a preceded global molecular level. Genome wide microarray enables the analysis of hundreds to thousands of genes simultaneously and such technology can identify particular specific patterns of gene expression or be used to identify candidate genes involved in the cellular event under study (Kerr & Churchill, 2001; Verducci et al., 2006).

2.7.1 Gene expression and microarray

Following the transcription of DNA into messenger RNA (mRNA), detection of these molecules, or their resulting protein products, signify that a gene is expressed in a cell or a group of cells studied. Microarrays are used to achieve this and are available in a large variety, as in house manufactured or customised arrays. All aim at determining or quantifying the expression levels of genes and, depending on the platform used, a certain set amount of genes intrinsic to the design of the array can be evaluated. Additionally, a large component of statistical input is necessary to selectively capture the specific intensity level of expression of the gene itself (Verducci et al., 2006). Comparative gene expression measurements and evaluations are the most common experiments in which mRNA levels are monitored in comparisons from tissue to tissue, cell to cell or temporally, such as different time courses (Kerr & Churchill, 2001; Verducci et al., 2006). An example of a comparative experiment would include the determination of gene expression differences observed between tumour cells and their counterpart normal cells. Another example would be the determination of differences from cells obtained from a specific organ of a genetically modified organism compared to the same cells from a non-genetically modified counterpart. The approach of analysis of differences of cellular gene expression is therefore very powerful, capturing the expression difference signature required to comprehend cellular functions. This applies to exercise science research, for which one could investigate the alteration in expression occurring between diverse sessions of exercise following a training intervention (Mahoney et al., 2005). In a time course experimental design, cells are collected at different time-points and commonly sampled before, during and after a training intervention (Churchill, 2002). Temporal gene expression analysis performed by microarray technology represents datasets that reflect cellular expression snapshots of the biological functional status of the samples studied. Statisticians and bio-informaticians are attempting to elucidate the underlying transcriptomic processes of all this data to reflect the physiological event being captured. Several approaches are attempted to elucidate the right pipeline to extrapolate relevant data. Nowadays, it is still a challenge to draw full conclusions, even if candidate genes are found. Post validation experimentations are always of utility to demonstrate if the researcher is not facing a false positive. On a larger scale of comprehension of the data resulting from microarrays, the typical gene set annotation approach provides angles of reflection on what the data attempts to reveal. While

microarray gene expression analysis offers the possibility to describe the different cascades of gene expression as a result of specific stimuli or to reveal the expression of tens and thousands of genes in other experimental types of settings, it is still agreed that more statistically and bio-informatically novel methods of analysis are required. New attempts to analyse large biological datasets are being put in place such as sparse partial least square discriminant analysis (SPLS-DA). This type of analysis focuses more on the most relevant and meaningful variables within the array data and has a better interpretability of biological data resulting from the thousands of variables observed in microarray data. However, the technology of microarray gene expression represents with no doubt an immense progress in the field of molecular biology and has revolutionised our understanding and perceptions of cellular gene expression profiles (Mahoney et al., 2005).

2.7.2 DNA to RNA and RNA to Protein

Cells contain nuclear and extra nuclear deoxyribonucleic acid (DNA) molecules that are the blue print information that is coded to direct and guide the development, functioning and behaviour of the organism (Snustad & Simmons, 2006). The DNA molecule detain this information in encoded sequences of nucleotides which are appropriately packed in the cell nucleus and comprise the genome (Snustad & Simmons, 2006). The genome comprises all of an organism's DNA (Snustad & Simmons, 2006), a small portion of which is coding DNA with an approximate of 20,000-25,000 genes. The genome is made of several types of sequences, including both coding and non-coding DNA. In the past, the non-coding DNA was wrongly named 'junk DNA' but progress in molecular biology and the cost effective use of technologies such as sequencing has provided a totally different interpretation of the role of non-coding DNA. The approximate 20,000-25,000 genes are regulated on several levels, some of which result from the role embedded in the non-coding regions of the genome. Importantly, most genes contain the coding instructions for the synthesis of primary polypeptide proteins, a succession of amino acids bonded together in chains. Proteins have characteristic structural sequences which can be evolving from primary to secondary, tertiary and possible quaternary structures to undertake their biological function (Snustad & Simmons, 2006). Coding regions within a gene are codons (triplets of adjacent nucleotides) that specify these sequences of amino acids (Snustad & Simmons, 2006). The synthesis of a

protein, directed by the information encoded within a gene, relies on expression stages, which are required to turn the DNA of a gene into a protein. Two major stages are required and are denoted transcription and translation. During transcription, an enzyme called RNA polymerase II is responsible for the copying of the DNA template into a pre messenger ribonucleic acid (pre mRNA) molecule. To create the RNA molecule, the polymerase copies the template by base recognition and addition of complementary bases (U complementary to A; C complementary to G; A complementary to T and G complementary to C) within an elongated RNA molecule (Snustad & Simmons, 2006). The copied RNA (the transcript) separates from its DNA template, forming the pre-RNA, which is then altered by post-transcriptional modifications, such as the deletion or addition of nucleotides. The altered product becomes the messenger RNA (mRNA) which encloses the encoded information that will be directed towards a genetic code aiming at the synthesis of a polypeptide in translation (Snustad & Simmons, 2006).

During translation, a template of the polypeptide is synthesised by means of inter- relational translation machinery that involve other types of non-coding RNA such as transfer RNA (tRNA) and ribosomal RNA (rRNA). Codons within exomic parts of the mRNA molecules are decoded within ribosomes and recognised by tRNA. All codons in mRNA represent the exomic parts embedded in the corresponding DNA and will be translated with each codon specifying an amino acid to be incorporated into the nascent polypeptide chain. When the translation process is finished, the polypeptide dissociates from the translational machinery and undergoes a series of post-translational modifications such as protein sorting, ultimately being modified to undertake its role in the cell (Snustad & Simmons, 2006).

Gene expression is essential for the cell to survive. It includes the transcription and translation of housekeeping genes whose functions are central to the survival of every single cell. On the other hand, genes that are functionally important are expressed for a particular cell type. When specific genes are required for a special function, their expression is mediated via an intracellular signal transduction pathway. This level of control ensures that gene products are produced only as required in quantities to closely satisfy the cellular function sought (Snustad & Simmons, 2006). These signal transduction pathways, which can be activated either extracellularly or intracellularly, are complex, often integrated with

multiple biological systems and involve proteins and a series of modifications of the cell and its content. Ultimately such signalling and cascades provoke changes or maintenance of biological functions such as the changes in skeletal muscle metabolism and microvasculature subsequent to exposure to stressful exercise conditions (Gibala et al., 2009). An example of a signal transduction pathway known to be activated by exercise is the mitogen-activated protein kinase (MAPK) pathway. These signalling cascades integrate intracellular signals from diverse extracellular stimuli, including exercise, growth factors and/or cellular stress, to regulate and adapt gene transcription and protein synthesis to various cell systems (Cohen, 1997; Yu et al., 2003).

At least three parallel MAPK signalling cascades, including extracellular regulated kinase (ERK1/2; p42/p44 MAPK), p38 MAPK and c-jun NH_2-terminal kinase (JNK) are activated in skeletal muscle in response to exercise, a physiological form of stress, in both moderately trained and untrained subjects (Yu et al., 2003). Furthermore, MAPK substrates including mitogen- and stress-activated protein kinase (MSK1, MSK2, p90 ribosomal S6 kinase, p90rsk, also known as MAPKAPK1) and MAPK-activated protein kinase 2 (MAPKAPK2) increase activity during exercise (Krook et al., 2000).

Once a stimulus has signalled gene expression to occur, the process of transcription will commence.

Figure 8 – A traditional view of gene expression

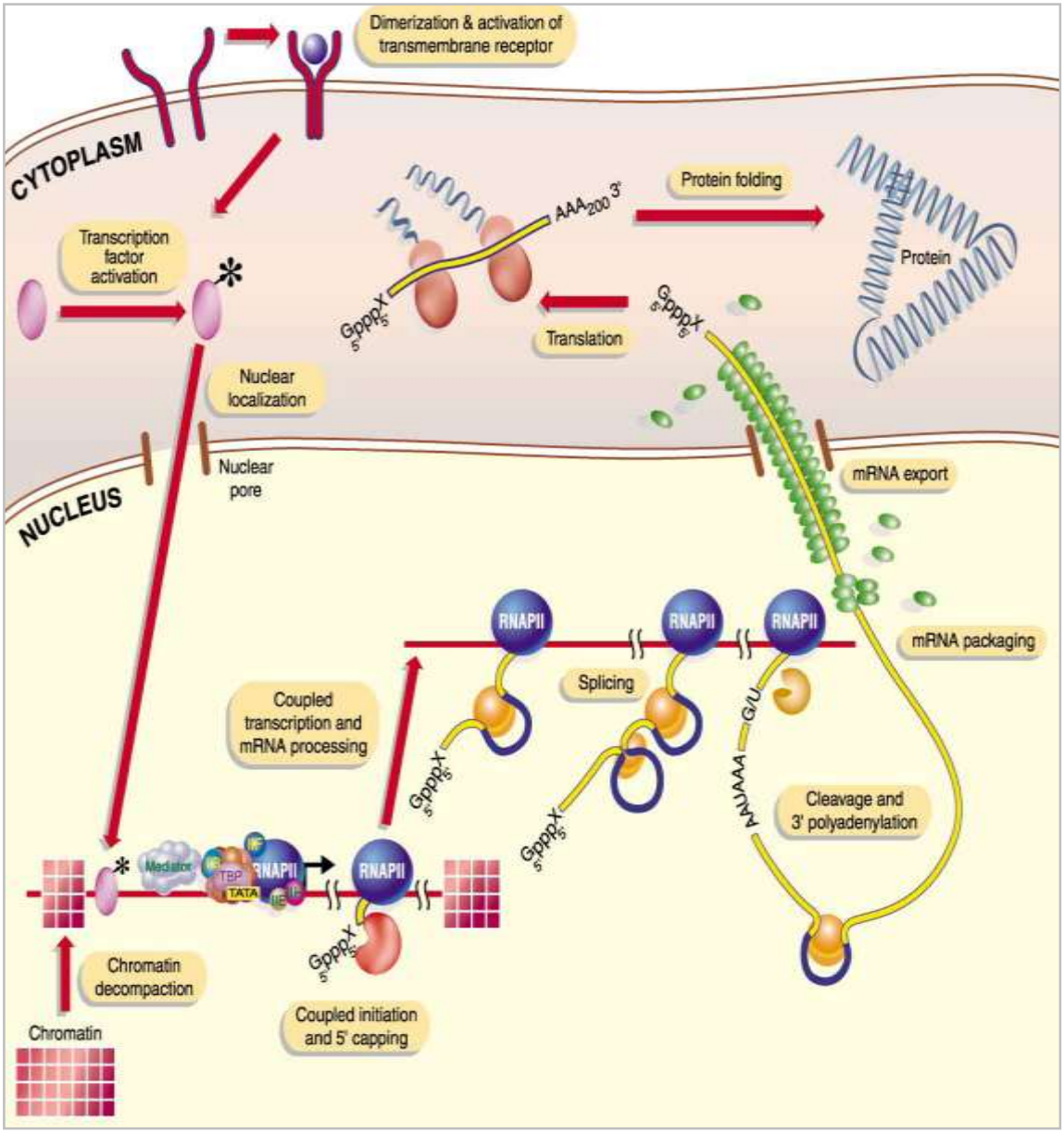


Fig8_(Orphanides & Reinberg, 2002). Illustration of the processes required for a gene to be expressed through the processes of transcription and translation. The enzyme RNA polymerase copies the template DNA in which the gene of interest resides. Transcription occurs in the nucleus and results in the formation of a pre-mRNA transcript. Following further maturation, the pre mRNA is modified to become an mRNA molecule due to mRNA splicing of introns and additions of a 5'cap and a 3'poly A tail. The mature mRNA molecule will undergo translation in the cytoplasm to build the corresponding protein with means of translation machinery.

2.7.3 Muscle tissue

Gene expression in response to exercise has been an emerging topic in the literature in recent years. Scientific attention paid to skeletal muscle tissue is associated with its role and mechanistic response to exercise, e.g. the changes in metabolism during endurance performance and power development for locomotion (Coffey & Hawley, 2007). Skeletal muscle has a powerful ability to adapt to stimuli, such as contractile activity and environmental stress (Coffey & Hawley, 2007). Different types of exercise stimuli or training, such as endurance and strength training, produce distinct differences in the musculature (Stepto et al., 2009). A study by Stepto et al. (2009) investigated global gene expression profiles in muscle tissue from individuals already involved in strength training and endurance training. In that study, these gene expression profiles were compared to the gene expression profiles of untrained subjects. The expression of genes related to mitochondrial and oxidative capacity was found to be the predominant signature of the endurance-trained group. The strength training group possessed no such signature (Stepto et al., 2009). It is important to state that the gene expression analysis performed by Stepto et al. (2009) used a spotted glass type microarray of 8000 gene spotted candidates and therefore did not represent the whole genome.

On that note, a study by Gibala et al. (2009) investigated the effect of an acute exercise session on six young men. The methodology consisted of four repetitions of 30-second sprints using a cycle ergometer, with four minutes recovery between sprints. Their hypothesis was formulated to discover whether acute intense intermittent exercise would activate signalling cascades by means of 5'adenosine monophosphate-activated protein kinase (AMPK) and the p38 MAPK pathway, as well as through the possible alteration of gene expression of PGC-1α, a protein associated with mitochondrial biogenesis in human skeletal muscle (Gibala et al., 2009). The researchers found significant changes in the signalling cascades and the expression of PGC-1α mRNA immediately after exercise and after three hours of recovery (Gibala et al., 2009). In a recent study carried out by Neubauer et al. (2014) the researchers examined PGC-1α and found similar results to that of Gibala (2009). Neubauer et al. (2014) used a whole genome microarray based technology to analyse several muscle biopsies from eight individuals (baseline biopsy obtained a week before a 2 hour endurance exercise trial); 3 hours post exercise biopsy; additional 48 hours

and 96 hours post exercise biopsies). PGC-1α gene expression was up regulated at time-point 3 hours post exercise with a fold increase of 10.3. This data confirms the previous findings reported in the study of Gibala et al. (2009). Additionally, interesting data resulting from these Illumina based HT12 v3 microarrays revealed an up regulation in gene expression of several other genes at time-point 3 hours post exercise. These genes consisted of Jun B Proto-Oncogene (JUNB), Chemokine (C-C Motif) Ligand 2 **(**CCL2) and Heme oxidase isoform 1 (HMOX1), which were found to be up regulated in expression by 3.8-fold, 5.0-fold and 15.1-fold respectively. Cytosolic messengers such as Ca^{2+} and AMP can explain this as they activate signalling cascades, creating alterations in gene transcription (Ntanasis-Stathopoulos et al., 2013). This gene expression alteration is caused by the activation of Ca^{2+}/Calmodulin dependent protein kinase (CaMK) and AMPK (Jørgensen et al., 2006). Furthermore, it has been suggested that AMPK could increase the expression of GLUT4 protein through the PGC-1α pathway (Michael et al., 2001; Baar et al., 2003).

It has been demonstrated by other researchers that muscle contraction creates a temporary response in mRNA that peaks 3-12 hours post exercise and, within 24 hours, returns to base line levels (Bickel et al., 2005; Pilegaard et al., 2000). Gibala et al. (2009) concluded that signalling cascades affected mRNA expression of PGC-1α, which would explain the increased mitochondrial biogenesis and increased capacity for glucose and fatty acid oxidation (Gibala et al., 2009). The results of the latter study might be explained by multiple bouts of exercise stressing the skeletal muscle, leading to an increased demand for ATP and thus favouring an expression of key genes in metabolic systems (Pilegaard et al., 2000). This will lead to an adaptive phenotype through increased mitochondrial biogenesis, an increased lactate buffering capacity and an increased ability to activate, transport and oxidise lipids (Pilegaard et al., 2000) leading to increased endurance performance.

Interestingly, acute exercise in sedentary individuals causes a hypomethylation of the skeletal muscles whole genome (Ntanasis-Stathopoulos et al., 2013). This hypomethylation promotes expression of metabolic genes such as: PGC-1α, TFAM, PPAR- δ, PDK4 and citrate synthase (Ntanasis-Stathopoulos et al., 2013). Interestingly, hypomethylation has been found to be exercise intensity dependent (Ntanasis-Stathopoulos et al., 2013), where an expression reduction in the methylation promotion of genes such as PPAR- γ, PGC-1α, TFAM,

PDK4 and MEF2A occurs immediately after high intensity exercise, along with a reduction of PPAR-δ 3 hours after high intensity exercise (Doi et al., 2009; Barrès et al., 2012).

To date, no transcriptional pattern is available that reflects the optimal training regime of exercise for specific performance outcomes. However, researchers have reported candidate genes in response to one single bout of endurance exercise. Pathway analysis revealed that these genes were involved in mitochondrial biogenesis, metabolism, cell growth and inflammation (Goffart & Weisner, 2003; Mahoney et al., 2005; Noonan et al., 2008). It is evident that physiological changes are the direct result of changes in gene expression. Thus, if specific gene expression levels can be found in response to certain types of training, marker genes in particular biological systems (e.g. metabolism) could potentially be identified and, in turn, used to measure and monitor training status.

When investigating the changes in muscle tissue, muscle biopsy is essential. When performing a muscle biopsy the overlying skin is anesthetised, an incision is made and sampling is performed through that incision with a 5-mm Bergstrom needle (Zeibig et al., 2005) or similar fine needles for biopsy procedures. However, it is clear that a muscle biopsy is an invasive procedure and non-invasive alternatives should be pursued for further research.

2.7.4 White blood cells

Examination of exercise induced gene expression changes in human leucocytes may act as a more accessible source of tissue than skeletal muscle. This in turn may provide a most precise and highly practical avenue for monitoring training status as described above, providing marker genes can be identified. This process would thus allow the highly abundant leucocytes to serve as a means for monitoring and evaluating an individual's biological response to exercise.

Microarrays have become a widely used tool in measuring the human leucocytes transcriptional response to exercise and have been found to be effective for simultaneously investigating gene expression levels in hundreds to thousands of genes (Zieker et al., 2005a). Using the microarray analysis tool, one has the possibility to investigate genome-wide

changes in a systematic way and possibly discover patterns of gene expression or even identify novel marker genes for monitoring responses to a specific stressor such as exercise (Zieker et al., 2005a). Using this method, scientists can gain a larger insight into the mechanisms involved in acute and chronic adaptation to exercise. Moreover, the marker genes reflecting these physiological changes may also serve as surrogate markers for exercise induced adaptations and modifications in tissues, such as skeletal tissue, potentially rendering muscle biopsy obsolete (Radom-Aizik et al., 2008). A preliminary study undertaken by Zeibig et al. (2005) investigated to what degree human blood cells would mimic the expressions in skeletal muscle in relation to exercise. They found that human blood cells mimicked some of the expression-profile alterations found in human muscle after endurance training (Zeibig et al., 2005). Some of the genes found to be expressed in both muscle and blood tissue after a longer period of endurance training were Carnitine Palmitoyltransferase 1B (CPT1B), Carnitine O-Acetyltransferase (CRAT), Glucose-Regulated Protein 58 (GRP58) and Organic Cation/Carnitine Transporter (OCTN2) (Zeibig et al., 2005). The researchers suggested that adaptation of oxidative metabolism in skeletal muscle might be reflected in the systemic processes in the blood. This has been further supported in another study by Lohninger et al. (2005) who investigated gene expression responses to endurance training and L-Carnitine supplementation in blood and muscle cells. In agreement with Zeibig et al. (2005) these researchers found significant changes in CPT1B, CRAT and OCTN2 in response to endurance training. It has been stated in the literature (Phillips et al., 1996; Jeukendrup, 2002) that endurance training for >31 days' increases fat oxidation at rest and during exercise. This is because muscle oxidative potential and glycogen sparing increase as a response to increased endurance capacity (Phillips et al., 1996; Zeibig et al., 2005). According to Jeukendrup (2002), increased glycogen sparing is one of the central adaptations to endurance training where the fatty acid oxidation in the muscle tissue increases and carbohydrate utilization decreases. The fatty acid oxidation rate between trained and untrained subjects depends on the expression levels of carnitine palmitoyl transferases 1 and 2 (CPT1, CPT2), which are responsible for enhanced transport of fatty acid into the mitochondria (McGarry and Brown, 1997; Sidossis et al., 1998). Several authors (Pilegaard et al., 2000; Hawley, 2002a; Tunstall et al., 2002) have identified an increase in mitochondrial protein concentration as well as an increase in the activity of CPT1 mRNA expression as a response to training. The authors identified these changes along with

increases in capillary supply as a positive response in the muscles of trained individuals (Pilegaard et al., 2000; Hawley, 2002a; Tunstall et al., 2002). In contrast to CPT1, which transport medium and long chain fatty acyl chains into the mitochondria, CRAT transports short chain acyl groups (Lohninger et al., 2005). In relation to energy homeostasis and fat metabolism, CRATs are important enzymes due to their distinct properties in relation to intracellular location and physiological function (Lohninger et al., 2005). Based on the findings of Zeibig et al. (2005) and Lohninger et al. (2005) that the expression of CPT1B, CRAT and OCTN2 are altered and positively correlated in WBC and muscle cells, it is suggested that they have a common mechanism of induction.

A study by Büttner et al. (2007) indicated that different regimes of exercise affect the leucocytes in a different manner, which can be effectively detected by microarray analysis. Notably, they found that gene expression levels changed in an intensity-dependent manner, as well as discovering novel expression changes in genes previously unknown to be affected by exercise (Büttner et al., 2007). In relation to the specific genes found to be regulated by exercise, there is variation between research findings from different studies (Zeiker et al., 2005a; Büttner et al., 2007). However, the validity of these differences cannot be confirmed, as the conflicting studies were heterogeneous in relation to sampling points, RNA preparations and microarray platforms. Again, different technical designs in the research may account for the variation in genes affected by exercise. These varying results may make the comparison of the exercise responses challenging, however by using the same microarray platforms one could standardise the analysis of results and in turn validate research findings against other standardised research. Regardless of minor limitations, WBCs presents a novel and accessible possibility for monitoring the molecular response to exercise (Table 5). Furthermore, due to this readily attainable, less-invasive tissue, athletes and their coaches might be more likely to comply to multiple sampling procedures during a season in order to monitor performance. For this reason, broad based microarray studies should be performed to observe novel gene expression changes after exercise that can be compared with traditional physiological measures.

Table 5 - Studies investigating transcriptional responses using microarray

Study	N= number	Sex & health status	Intervention	Tissue	Measuring technique	Statistical method	Project design
(Büttner et al., 2007)	5	Male recreationally active	ET 80% of VO_2max & 60% of VO_2max. 2 wks. apart	WBC	Microarray	GCOS 1.1, GeneSpring 6.1. ANOVA W/Benjamin-Hochberg or Bonferroni algorithms	Blood was collected pre & 1h post. Reports Standard Deviation (SD)
(Connolly et al., 2004)	15	Male, Healthy	30 min approx.80% of VO_2peak	WBC	Microarray	Affymetrix MAS 5.0 software, Normalized and modelled with the dChip program of Li &Wong. Stat.comparison Cyber-T stat program	Blood collected pre, immediately post and 1h post. They seem to have replicates. Reports Standard error (SE)
(Radom-Aizik et al., 2008)	12	Male, healthy	30min of constant work on bike 50% of the difference btw AT & VO_2. Approx.80%	WBC	Microarray	Quantified & analysed by GCOS 1.4, w/default values. ArrayAssist version 4.0.3 (STRATAGENE) Normalization using GC-RMA. BRB-ArrayTools software version 3.4.1 to determine sig. changed probe	Pre and immediately post. Reports SE
(Radom-Aizik et al., 2010)	11	Healthy men	30min interval training w/intensity of approx. 75%VO_2	WBC	Microarray	Quantified & analysed by GCOS 1.4, w/default values. ArrayAssist version 4.0.3 (STRATAGENE) Normalization using GC-RMA. BRB-ArrayTools software version 3.4.1 to determine sig. changed probe	Blood collected pre and immediately post. Reports SE
(Zieker et al., 2005b)	8	Trained males	Marathon comp	WBC	Microarray	R 1.8.1 with a mixture of Bioconductor structures and self-written functions	Pre. Comp. Immediately post. After 1h & 2h Large Subject variation in age and training status

2.7.5 Low versus high throughput methods

Quantitative northern blot is a low-throughput method used in laboratories to determine gene expression levels. This technique consists of performing an electrophoresis of previously isolated RNA molecules from biological samples. RNA molecules that are negatively charged migrate to the anode and are separated through the gel pores according to size (Pfaffl, 2001). Once the electrophoresis is terminated, the gel containing the migrated RNA is applied on a nylon membrane, an immobilizing matrix, and a blotting is performed to transfer the RNA to the membrane (Pfaffl, 2001). To assess the presence of a specific gene, the corresponding RNA molecule is annealed against a DNA based labelled probe that is complementary to the molecule to be identified. This probe is incubated together with the blot under special conditions that will stimulate the annealing of both molecules (Speed, 2004). This process is called hybridization and is followed by a wash step aimed at discarding unbound excess of probes (Speed, 2004). The amount of blotted RNA that was hybridised is labelled proportionally to the amount of corresponding specific probes. In order to assess appropriately the level of expression of a candidate RNA molecule, a housekeeping gene is usually measured at the same time. A housekeeping gene is a gene whose expression is constant in a biological sample and whose level of expression is therefore relative to the quantity of the biological sample being interrogated. A relative gene expression assessment is therefore possible when other RNA candidates are measured (Pfaffl, 2001). Depending on the nature of the labelling chemistry being used to detect the probes, the resulting hybridisation of the RNA and the probe can be detected by different means. Detection can be undertaken directly through laser scanning, the use of a CCD camera or indirectly by exposing an X-ray film to the blot (Pfaffl, 2001). The intensity of the signal can be visualised and quantified as an estimation of the amount of the targeted RNA molecule (Pfaffl, 2001; Speed, 2004). Northern blot is a technique that has been used for a certain amount of time and remains a good tool for quantitative gene expression purposes (Speed, 2004).

An additional low-throughput method has been possible since the development of polymerase chain reaction (PCR). In addition, with the advances of other associated techniques such as real-time PCR and the incorporation of labelled molecules, a quantitative PCR of high sensitivity can be performed (Pfaffl, 2004). PCR can be used to estimate the

concentration of a particular target RNA relative to a reference, a housekeeping gene (Pfaffl, 2004; Speed, 2004). Extracted mRNA or total RNA is converted to complementary DNA (cDNA) using the enzyme reverse transcriptase in a step called Reverse Transcription. PCR is then used to amplify exponentially a specific template (Speed, 2004). Specific oligonucleotide primer pair sequences are designed to target a region of a gene. The cDNA is denatured at 95^0C and the primers are added to the sample. At annealing temperature (50^0C to 65^0C generally), the two primers flanking the desired region to be amplified are annealed to their complementary regions in the template. These primers can then be used as a starting point for a DNA polymerase to copy the template gene, which constitutes the elongation step of the PCR cycle at 72^0C. Several cycles are then possible which, as a result, will amplify exponentially the targeted template. Primers targeting housekeeping genes are also used to normalise data as discussed previously within the northern blot section. Comparative expression is performed, as this analysis must take into account levels of the housekeeping gene expression (Speed, 2004).

Quantitative reverse transcription PCR can be measured by real-time PCR. These PCR runs do not measure levels of end- amplified products; instead they measure amplicon abundance during the exponential phase of the chain reaction (Pfaffl, 2004; Speed, 2004). This method is a more efficient method but requires an equal PCR efficiency between target genes and housekeeping genes (Pfaffl, 2004; Speed, 2004). In this technique the target and reference sequences are amplified and detected in the same real-time PCR instrument (Speed, 2004). Commonly, the amplification by PCR is associated with the introduction of a fluorescence marker. Such a marker is incorporated by the DNA polymerase after each cycle and accumulates with the PCR product. Real-time PCR instruments have a set of lasers that can excite these fluorescent probes and the emissions of the molecules are detected by the machine “in real-time”. Computation of the fluorescent emission following each cycle is visualised and therefore the user captures the dynamic features of the PCR in real-time. As PCR is meant to be exponential, the user has to be able to gain data in the exponential phase of these PCR runs. In reality, PCR is subject to several limitations and exponential growth is indeed not sustained over time as the reaction plateaus, for example when DNA polymerase activity is reduced or when reagents are depleted over time. A threshold line is placed by the user on the computer screen that is aimed at intersecting the fluorescent

curves exactly at the exponential phase of the PCR. This precision of placement of this threshold line is possible by logging the PCR curves, as only the exponential portions of the PCR would be visualised linearly once that log is applied. The user can therefore safely place this threshold line with this simple quality control positional detail. The intercept of the threshold line with the PCR curve denotes the Ct value (Cycle threshold). A cycle threshold is the cycle that corresponds to this intercept. Real-time PCR has a large number of different protocols and different instruments for performing these different assays (Speed, 2004). Primers flanking one gene can be used to amplify its target in two different biological samples. Therefore, two PCR curves can be obtained and two CT values computed where their differences are represented as the delta CT for the gene. If these two biological samples are also interrogated by a primer set of a known housekeeping gene, another set of CT values are computed specific to the housekeeper, as delta CT for the housekeeping gene. Calculation of the between the two different delta Cts corresponds to the delta delta Ct. 2^{-DDCT} represents the fold gene expression difference of the targeted gene between the two biological samples.

Comprehensive analysis of gene expression patterns can be obtained through several other techniques when high-throughput methodology is used. Serial analysis of gene expression (SAGE) is a quantitative technique used to assess gene expression levels (Velculescu et al., 1995; Man et al., 2000). The technique is based on PCR and sequencing but does not require per se a hybridization technique to assess gene expression. The SAGE methodology is based on the principle that the mRNA of transcribed genes holds short oligonucleotides that are specific to that particular transcript. These mers of 14 nucleotides are short sequence tags derived from a defined position within a transcript and contain sufficient information to uniquely identify the transcript (Chen et al., 1998; Kal et al., 1999). Biological samples are used to extract mRNA and subsequent cDNA synthesis is undertaken from these transcripts using biotinylated primers. This pool of cDNA is then immobilised on streptavidinated beads that will anchor the transcriptome cDNA. An elegant library preparation is then performed that will first shorten all anchored cDNA molecules using a restriction nuclease enzyme. This step is followed then with a second but different restriction that releases shortened cDNA from the beads and PCR based primers are allowed to attach to this cDNA. Ditags and

concatemers can be generated following this same procedure and PCR can be therefore undertaken to produce an elevated amount of material to be sequenced.

Following sequencing, the procedure then transitions towards a counting-based analysis of specific tags in which each tag among several thousands are numbered. Their count is proportional to the amount of the specific transcript, which enables a precise estimation of gene expression of each transcript. This simple measure of gene expression levels of many transcripts simultaneously can also reveal additional data beside gene expression levels. SAGE technique is capable of revealing polymorphisms existing in transcripts since sequencing can capture subtle nucleotide differences. In addition, this technique can reveal information on unknown genes as tags of these unknown genes will also be counted and captured. SAGE offers a cost-effective and inexpensive technique capable of examining the entire transcriptome of thousands of expressed genes in a cell (Zhang et al., 1997; Chen et al., 1998; Kal et al., 1999; Man et al., 2000). To investigate a comparative gene expression of two biological specimens, the typical SAGE experiment would start with two sources of mRNA populations to compare. For each tissue, a library of 10,000 – 50,000 tags would be generated by using the SAGE protocol mentioned above. Unique transcriptome tags would be counted and computed with frequency of unique tags compared between the two samples. For the tags, which are significantly differentially counted between the two tissues, a conclusion can be drawn that the corresponding genes are differentially expressed between the two sources of RNA (Audic & Claverie, 1997; Man et al., 2000).

Additional high-throughput gene expression methods are based on microarray technology. Commonly, several types of technologies are used and include glass-based arrays, nylon membrane based arrays, spotted based arrays and high-density oligonucleotide based arrays. The nylon membrane array has been widely used around the world and is one of the oldest array technologies (Meier-Ewert et al., 1998). PCR based transcripts are generated purified and arrayed on Nylon membranes. Following protocol based baking and quality control checks, these typical filter based microarrays contain 600 – 2400 bases in length spotted into grids on the surface of the membrane (Meier-Ewert et al., 1998). Target cDNA, derived from mRNA extracted from the biological samples of interest, is labelled with radioactive probes and allowed to be hybridized to the array. The level of hybridisation is

dependent on the abundance of the corresponding transcript and the filter is then exposed to X-ray film for subsequent visual imaging. As a result, a digital image can be obtained and considered as the experimental raw data to analyse (Meier-Ewert et al., 1998).

With spotted microarray technology, oligonucleotides or cDNA products corresponding to specific genes are spotted on glass slides or can be already available when synthesised in chip platforms available through manufacturers. During spotting, a robot spotter normally performs the process in which each gene can be represented by different but specific DNA fragments (Schena et al., 1995; Hughes et al., 2001). Contrary to oligonucleotide arrays, spotted arrays are "customizable"; the user can choose which probes to be spotted according to his or her specific experimental needs. These arrays are usually hybridized with labelled cDNA resulting from the reverse transcription of extracted RNA from biological samples. Double channel hybridization experiments are chosen to distinguish, by means of different labels, the two sources of RNA populations: Two cDNA samples can therefore be generated and labelled with two different fluorophores (e.g. Cy3 and Cy5). Once clear specific labelling is undertaken, both distinct cDNA samples are mixed and left to hybridize to the platform for an optimal time and under stringent conditions. The core principal of this hybridisation is to provide enough time for each set of cDNA to bind competitively to their immobilised complementary target. A series of washing steps is then applied to the preparation to attempt in the removal of any unbound material or unspecific binding that may have occurred. The platform is then subjected to scanning using corresponding wavelengths necessary for the excitation of the fluorescent dyes previously used in the experiment. The possibility to distinguish both spectral fluorescent emissions of the two hybridised samples is now possible as ratios of dye excitation intensities are analysed (Schena et al., 1995; Hughes et al., 2001). Such analysis though is preceded by a series of normalisations within the array itself or/and between arrays if several were run. Through careful normalisation, ratios of intensity between both emitted fluorescence can be calculated and fold expression differences of every single spotted gene can be assessed.

Different technologies have evolved rapidly to enable a higher representation of material immobilised on array platforms. This permits the user to undertake their experiment with

significantly lower amounts of starting material to be hybridised. In addition, thousands and thousands of short oligonucleotide probe pairs can nowadays be 'printed' on small glass chips that are synthesised to be specific to a certain gene (Speed, 2004). This high-density oligonucleotide arraying technology is progressing fast and permits not only a higher representation, up to 20 probe pairs, of a single gene but also can distinguish, with no ambiguity, closely related gene sequences. This level of representation and specificity generates data of high quality, providing users with the possibility of evaluating the expression of thousands of genes in a single run. Quality controls and assessment of fluorescent intensities are performed to conclude on transcriptomic differences between particular biological samples (Fodor et al., 1991; Lockhart et al., 1996; Speed, 2004).

As previously described, several techniques are available to investigate profiles of gene expression at the transcriptome level. Experimental design is primordial to obtaining meaningful datasets surrounding hypotheses and questions to be asked and answered (Kerr & Churchill, 2001). Experimental constraints are numerous such as the cost of reagents, the sample size of the study and the integrity of extracted mRNA. Additionally, proper use of data-mining tools is essential to explore relevant patterns of expression and the possible relationships between candidate genes (Kerr & Churchill, 2001). As previously introduced, normalizing the data is essential before obtaining meaningful datasets that determine as accurately as possible the cellular expression levels within the samples under investigation. Fold expression changes usually obtained by logarithm base 2 transformations enable the researcher to investigate all sorts of information including altered physiological pathways in disease, gene profiling resulting following the action of a stimulus, and temporal responses to a physiological change. In other words, regardless of the project performed, one common goal for researchers is the identification of genes that are differentially expressed between one or more pairs of samples (Quackenbush, 2002). In the first instance, reducing a data set to genes that are most variable between samples is of interest but does not necessarily answer the initial hypothesis. In the past, a fixed fold-change-cut-off of generally two fold was used to identify the genes with most variation (Quackenbush, 2002). However, more recently, calculation of the mean and standard deviation of the distribution of log2 (ratio) values enabled a better global fold-change difference and confidence to be defined

(Quackenbush, 2002). However, a flaw has been identified with this method, as this approach may not reflect the essential structure in the data accurately and is common at low intensity reading levels (Quackenbush, 2002). Of note, subtle changes of expression levels for genes, such as transcription factors known to induce expression of the vast majority of genes in time, would not be captured if outside the fixed fold-change-cut-offs.

Use of the t-test, which is the most popular analysis test, has been a matter of discussion in the literature. However, computing a t-statistic can be problematic as genes having a low variance can skew variance estimates. These genes are falsely selected as differentially expressed as they are associated with large t-statistics (Audic & Claverie, 1997). Furthermore, another drawback comes from its application on small sample sizes, which implies low statistical power (Eisen et al., 1998). As a result of this, questions have been made of the importance and the efficacy of the t-test, along with the importance of variance modelling (Golub et al., 1999). In the hope of creating improved variance estimations of accuracy and power, the development of many innovative alternatives has been created. Although these alternatives appear varied at first glance, they fall into a few nested categories dependant on both statistical and biological hypotheses such as: parametric or non-parametric modelling, frequentist or Bayesian framework, homoscedastic hypothesis (same variance between groups of samples) and gene-by-gene variance estimation. However, as each strategy operates under specific assumptions, different tests generally identify a different list of significant genes (Irizarry et al., 2003). Additionally, though a prosperity of available methods exist, the t-test remains widely used in gene-expression studies. This might be due to its simplicity and interpretability in nature. Although researchers seem to have a tendency to use the t-test, identifying which approach is the most appropriate to use in analysis of gene expression data remains a crucial issue.

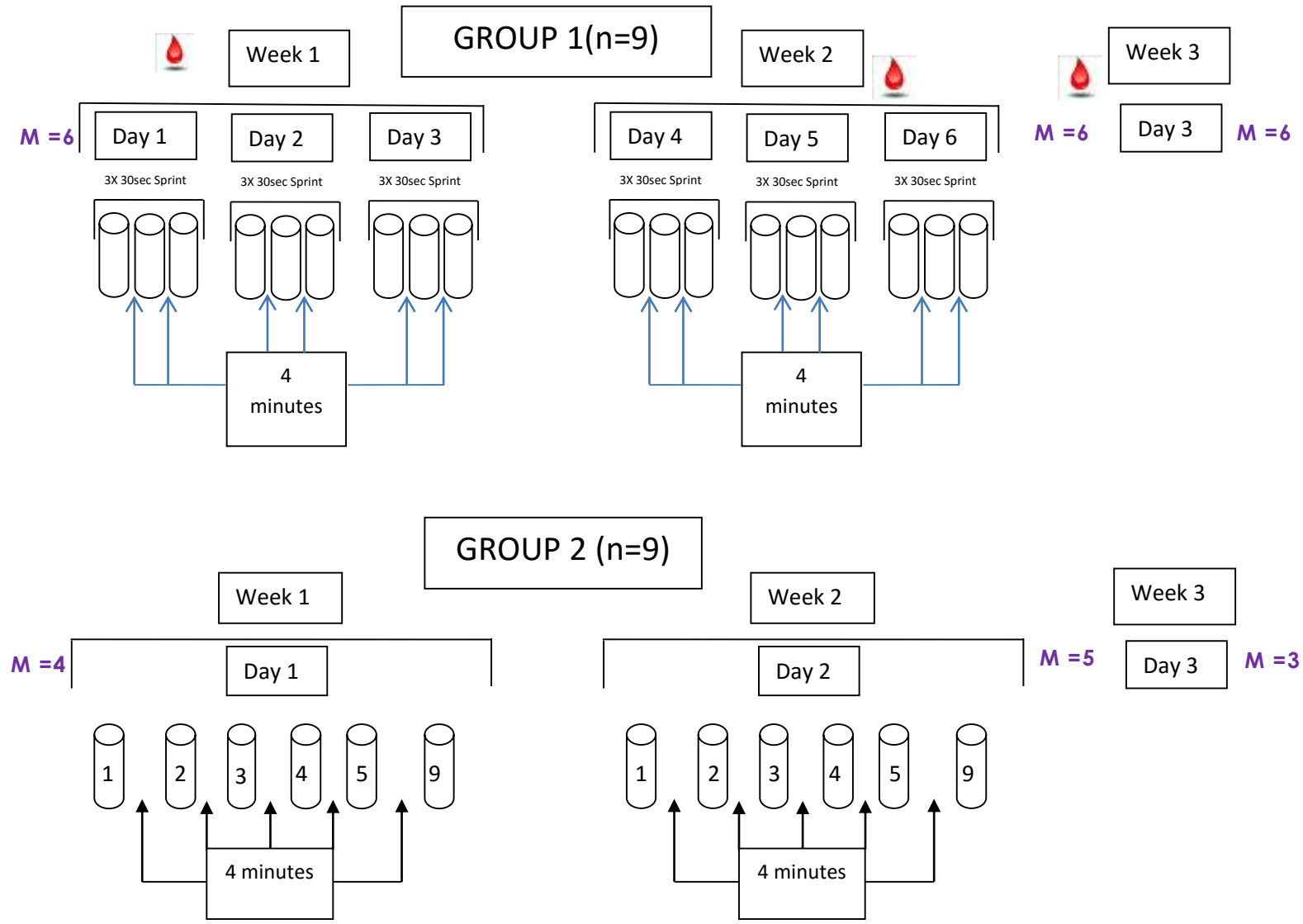
Chapter 3 - Methodology

3.1 Training intervention

The training intervention schedule is shown as a diagram below (Figure 9). Group 1 came in for training three times per week on Mondays, Wednesdays and Fridays for two weeks. At each training session, group 1 performed 3 repetitions of 30-second all-out sprints. Group 2 came in for training once per week on Wednesdays for two weeks. At each training session, group 2 performed 9 repetitions of 30-second all-out sprints.

Each training session consisted of repeated 30-second all out efforts on an electronically braked cycle ergometer (Lode) against a resistance equivalent to 0.075 – 0.095 kg/kg body mass. After a 5-10 min warm up, there was a 2 s “lead in” where the subjects were instructed to begin pedalling “as fast as possible” before a computer interfaced to the ergometer applied the designated load. The subjects were verbally encouraged to continue pedalling as fast as possible throughout the 30 s test. Peak power, mean power and fatigue index were subsequently determined. During the 4 min recovery between each 30 s test, the subjects remained on the ergometer and continued cycling at a low cadence.

Figure 9 - Training intervention schedule for G1 and G2



Legends	Denotation
	Microarray (**M**) experiments Samples were collected at three time-points both in group 1 and 2 as follows: -at Pre week 1 -at immediately post week 2 -at 3 days post week 2
	Sprint of 30 seconds (electronically braked cycle ergometer (Lode) against a resistance equivalent to 0.075 – 0.095 kg/kg body mass)
	Blood collected: -at Pre week 1 -at immediately post week 2 -at 3 days post week 2

3.2 Subject characteristics

Trained cyclists aged 18-35 years old (n=26) were randomly assigned to either group 1 (n=9) or group 2 (n=9) and were required to experience a 2-wk sprint interval training intervention. A control group (n=8) was not required to perform any training intervention. However, all control subjects and trained participants were assessed by VO_2max, ECT and Wingate performance tests. Following a routine medical screening prior to the study, all groups and controls participant were informed about & familiarized with the procedures and protocols used in the study. Bond University Human Research Ethics Committee (BUHREC) approved this whole project; ethics number RO1063.

3.2.1 Subjects

Trained cyclists aged 18-35 years old (n=26) were recruited to take part in the study. After a routine medical screening, all subjects were informed about the protocols to be used in the study and all associated risks were outlined. The participants were required to give their written consent before taking part in any measurements and complete a familiarization session with the protocols and equipment, including the mouthpiece with nose-clip and the facemask.

Table 6 - Characteristics for participating subjects (N= 26)

Age (yrs.)	32.0 ± 6.6
Height (m)	1.80 ± 0.05
Weight (kg)	78.8 ± 6.2
VO_{2peak} ($mL \cdot kg^{-1} \cdot min^{-1}$)	49.5 ± 6.3
VO_{2peak} ($L \cdot min^{-1}$)	3.9 ± 0.4
Average training sessions per wk.	4 to 5
Average intensity of training sessions	Moderate to heavy*

*Moderate to heavy = Subjects subjective measure of their own training sessions (Light, moderate or heavy)

3.2.2 Dietary and Exercise Control

In an attempt to minimize any potential diet and training-induced variability, all subjects were instructed to keep their diet and training regime similar throughout their involvement in the project. The subjects were instructed to record all dietary intake as well as training sessions throughout the whole project and compliance was assessed by performing a dietary and training analysis on the individual food and training records maintained by the subjects. This was quantified through a diary, designed specifically for this project, where each subject recorded in detail their dietary intake and training sessions throughout their involvement in this study (See Appendix 6).

3.3 Protocols, equipment and software

3.3.1 Cycle ergometer

Cycle ergometers enable the precise measurement of work rate in exercise testing protocols. Advantages of the cycle ergometer over the treadmill include the ability to vary the work rate in step, incremental, or ramp fashion; the ability to determine work efficiency; potentially greater safety because the subject is supported at all times; and less movement artefact on measurements. On the other hand, the seat may become uncomfortable during a long testing or training session, which may have an effect on the exercise time. In the present study an electronically braked cycle ergometer (Excalibur Sport V2, Lode, Groningen, The Netherlands) was used. The Lode ergometer uses a variable electromagnetic field to produce a braking force to pedalling that varies with flywheel speed, changing the resistance to cycling to maintain work rate at a set level essentially independent of pedalling speed.

3.3.2 Cardio-pulmonary testing equipment and software

Although exercise testing can be performed with little or no equipment, the more sophisticated and potentially useful analysis of cardio-pulmonary function during exercise necessitates gas exchange measurements. A variety of systems, measuring devices,

recorders, and other equipment have been put together for these purposes. There are now commercial systems that determine gas exchange using either a gas-mixing chamber or breath-by-breath analysis of expired air.

A breath-by-breath system, as used in the present study, measures airflow or volume continuously, while simultaneously determining instantaneous expired CO_2 and O_2 concentrations. The CO_2 output and O_2 uptake during each breath are calculated and the cumulated totals of all breaths over a measured time period are reported as VCO_2 and VO_2. Breath-by-breath systems make it possible to determine gas exchange rapidly, accurately and under many conditions. By interpolating breath-by-breath expired volume, O_2 uptake and CO_2 output second by second, it is not only possible to reduce the variability in breath-by breath measurements, but also enhance the measurement of physiological responses to rapid changes in work rate.

In the present study, the MedGraphics Ultima PFX equipment and software was used. This is a compact pulmonary function and exercise system that is capable of measuring all standard pulmonary function and gas exchange variables. This system is designed around the preVent^{TM} pneumotach, which provides accurate flow and volume measurement. The system measures complete spirometry, single breath diffusion, single breath and multiple breath nitrogen washouts. In addition to pulmonary function testing, the ultima PFX can also perform cardio-pulmonary exercise testing, by collecting breath-by-breath measurements of flow, O_2 and CO_2 with other calculated gas exchange parameters.

3.3.3 Wingate - Anaerobic Test

The Wingate Test (WAnT) is a maximal intensity cycle ergometer test of 30 s duration. The WAnt was developed during the 1970's and serves to evaluate anaerobic performance (Beneke et al., 2002). The WAnT is widely used and is considered the most popular test of anaerobic muscle performance (Bar-Or, 1987). The WAnt requires the subject to cycle at their maximal speed for 30 seconds against a pre-determined braking force. The braking force is kept constant throughout the test, but because the magnitude of braking force relative to body mass is high, the peak pedal cadence cannot be maintained for more than a few seconds before the subject begins to fatigue and reduce pedal cadence. The mechanical

power during a WAnT is measured using the equation: **Power = force x velocity** (Bar-Or, 1987). Force equals the braking force (resistance) that acts on the perimeter of the Lode bike flywheel. The velocity is the distance travelled over time at the flywheel perimeter. Because the crank length is constant, the braking force (Torque in Nm) remains constant throughout the test and effectively the peddling speed is measured to calculate the power. The work is then calculated by: **Work = power x time** (P (w) = F (N) x $rev \cdot s^{-1}$ x 6 $m \cdot rev^{-1}$). WAnT requires a power level that in turn would require two to four times the participant's maximal oxygen consumption (Bar-Or, 1987). Thus the physiological basis of the WAnT is based on the contribution of the anaerobic pathway. A study by Green, (1995), determined that 30 s is an optimal duration for an all-out test of anaerobic work or mean anaerobic power. Thus, the first seconds of the WAnT rely predominantly on the phosphagen system (Green, 1995). However, total work during a 30 s WAnT requires energy production from both the phosphagen system and glycolysis (Green, 1995). Total work is based upon the total number of revolutions at the end of the 30 s test and the force setting against which the participants pedalled. The calculation of total work is the product of the force setting (N) times the total distance cycled (which is the number of revolutions cycled times 6 m per pedal revolution) **Total work (kJ) = Force (N) x Revolutions x 6m$\cdot$rev^{-1} / 1000** (Bar-Or, 1987). Furthermore, mean anaerobic power is a function of total work done divided by the time over which that work was completed (Bar-Or, 1987).

Mean anaerobic power mainly reflects the ability to transform energy from the anaerobic glycolytic pathway, while anaerobic capacity reflects the amount of energy that can be produced from the glycolytic pathway (Serresse et al., 1988; Bogdanis et al., 1996; Beneke et al., 2002; Minahan et al., 2007). Furthermore, there is a consensus through the literature that during a WAnT, 31 – 32 % of energy is derived from the ATP-PC system, 45-50.3 % from the glycolytic system and 18.6 - 21 % from oxidative metabolism (Serresse et al., 1988; Bogdanis et al., 1996; Beneke et al., 2002; Minahan et al., 2007).

The results of the WAnT test depend upon the braking force selected for each subject. If the braking force is too low the subject will be able to maintain a high level of pedal cadence during the entire test. The braking force on the WAnT is traditionally set by having the subject cycle at maximal pedal cadence speed for 30 seconds against a resistance related to

body weight (40g/kg bodyweight with a Fleisch ergometer; 75g/kg body mass with a Monark ergometer) (Vandewalle et al., 1987). Determination of the initial braking force can be challenging because the relationship between force and mean power is parabolic. The value of the braking force can be either standard (5.5kg with a Monark cycle-ergometer) or related to body mass (Vandewalle et al., 1997). In the latter case, a research group has produced different resistances [0.075 kg/kg bodyweight (Bar-Or, 1987); 0.087 kg/kg bodyweight (Dotan & Bar-Or, 1983)]. The low value of resistance proposed by Ayalon et al. (1974) on the Wingate test is probably explained by the fact that the subjects of their first study were children (Vandewalle et al., 1987). Furthermore, Dotan and Bar-Or (1983) collected WAnT measurements from 18 female and 17 male physical education students to determine the optimal resistance to produce the maximal power output for peak power and average power (see Table 7). As well, a Fleisch ergometer was used and the resistance for males that produced the highest average power was determined to be 52 $g.kg^{-1}$ BM (Dotan & Bar-Or, 1983). This load is equivalent to 0.87 $kp.kg^{-1}$ BM on a Monark ergometer (Dotan & Bar-Or, 1983). Accordingly, this study chose the constant defined by Dotan & Bar-Or (1983) in order to allow for larger athletes and to make valid comparison of WAnT among subjects of this study.

Table 7 - Constants for comparisons of WAnT between subjects

T (torque) = G (body weight) x c (standard).

Leg ergometry	**X body weight = Torque (in Nm).**
Male adult	0.75
Athlete male adult	**0.87**
Female adult	0.67
Athlete female adult	0.77
Boys (age 7-14)	0.55
Girls (age 7-14)	0.53

(Dotan & Bar-Or, 1983)

During the WAnT the selected performance measures were peak power, mean power and the fatigue index (Hopkins et al., 2001). Peak power is the highest mechanical power elicited from the WAnT taken as the average power over any 5 s period and is shown to correspond to maximal anaerobic power. Mean power is the average power maintained throughout the

six, 5 s segments as an index of anaerobic capacity. Finally, the fatigue index is defined as the rate of decline in power during the test expressed as a percentage of peak power (Beneke et al., 2002).

3.3.4 Maximum oxygen consumption - VO_2 max test

VO_2 reflects in part the O_2 utilization by the muscle cells performing the exercise. The VO_2 – work rate relationship describes the rate of O_2 utilized by the exercising muscles relative to the amount of external work performed. With prolonged exercise training, stroke volume adapts to significantly increase at rest and during exercise (Weiner & Baggish, 2012). While cardiac output, the product of stroke volume and heart rate, increases up to 5 - 6 fold during a maximal exercise effort (Weiner & Baggish, 2012). Oxygen uptake is the product of cardiac output and the arterio-venous oxygen difference (Weiner & Baggish, 2012). The relationship between cardiac output and VO_2 can be quantified through the Fick equation (cardiac output = VO_2 x arterial – venous O_2 difference), which suggests that there is a direct affiliation between the two (Weiner & Baggish, 2012).

All subjects performed an incremental exercise test to volitional fatigue on an electronically braked cycle ergometer (Excalibur Sport V2, Lode, Groningen, The Netherlands) to determine the VO_{2peak} using an open circuit spirometry system (Medgraphics). The incremental exercise test consisted of three stages; warm-up stage (50W for 5 minutes), an exercise stage (increasing power, 10W every 20 s until volitional fatigue) and a “cool-down stage” (power decreased to 50W for 10 minutes). The value used for the analysis of VO_{2peak}, was the highest value found after averaging the data for the last two 30-second collection periods of the incremental exercise test.

Many of the existing cycle exercise protocols have stages of two or three minutes in an attempt to produce a “steady state” of oxygen consumption at each stage. The time taken to reach a “steady state” is variable, and can depend on the physiological fitness capacity of the subject and the selected stage increments in work rate (Fairshter et al., 1983). Incremental exercise tests consisting of a few prolonged stages may result in the subject becoming familiar with the protocol during repeat testing and increases the possibility that

the subject could repeatedly stop exercising at the same stage. This learning effect may mask improvement with treatment (Meyer et al., 2004).

This study therefore designed a protocol with small, frequent increases in workload, as recommended by others (Fairshter et al., 1983). Such a protocol is useful if there is subsequent, sub-maximal exercise testing. During a VO_2max/peak test, there are three points, which are specifically used for performance measurement purposes. Different data plots representing the relations involving CO_2 production, O_2 uptake and minute ventilation can determine changes in gradient (see Figure 1) which reflect the anaerobic threshold (AT) or ventilation threshold 1 (VT1). A second sudden change in gradient can also be determined and refers to the respiratory compensation threshold (RCT) or ventilation threshold 2 (VT2) (see Figure 1). In Figure 2, a third point is representing VO_2max/peak. The AT and RCT are characterised by disproportionate increases in expired minute ventilation (V_E) in relation to VO_2 and CO_2 production (VCO_2) during incremental exercise (Cross et al., 2012). In practical terms for the athlete, when reaching AT the athlete will experience increased ventilation and a sensation of "heavy legs". Furthermore, at RCT the athlete will experience hyperventilation and intense fatigue. As the athlete reaches this RCT point, they will be seconds away from VO_2max (Meyer et al., 2004). At VO_2max, the body's metabolic demand has surpassed the body's ability to deliver sufficient oxygen to the working muscles. Such an amplitude of maximal oxygen consumption will cause the athlete to fatigue and end the test (Meyer et al., 2004).

3.3.5 Endurance Capacity Test (ECT)

The use of the ECT as opposed to a time trial (TT) has been well discussed in the literature. Both tests are used to investigate the effects of various exercise-training programs on endurance performance. A TT exercise test is traditionally defined as an endurance performance test with a known endpoint (Laursen et al., 2007). Subjects in TT tests are required to complete a set distance in as fast a time as possible, or complete as much work as they can within a given time period (Laursen et al., 2007). Furthermore, subjects performing a TT are usually made aware of the distance or duration of the TT so that they can adjust their work output to pace themselves towards this known endpoint (Laursen et al., 2007). Conversely, an ECT test is performed at a constant speed or power output until

volitional fatigue (Laursen et al., 2007). This type of protocol is advantageous to the researcher compared to the TT when assessment of exercise capacity at a steady rate of exercise is sought. TT tests allow for fluctuations of exercise intensity and for this reason add “noise” to the measured markers (Laursen et al., 2007).

Subjects cycled to volitional fatigue on an electronically braked cycle ergometer (Lode, Groningen, The Netherlands) at a power equivalent to an oxygen consumption that represented 60% of the difference between the first ventilation threshold (VT1) and VO_{2peak}, as modified from a study by DiMenna et al. (2009). All endurance performance cycle tests were performed in the absence of any temporal, verbal or physiological feedback. The cycle tests were terminated when the cadence fell below 40 rpm for 10 s and the exercise duration was recorded. Expired breath-by-breath air was measured to determine the oxygen uptake and related measurements and averaged over 30 second collection periods. Cycle endurance capacity tests were performed with a facemask. Medium and small facemasks were offered to all the subjects (Hans-Rudolph 7600 series V2 full face CPAP mask with headgear). The facemask was fitted over the subjects’ nose and mouth, using an elastic headgear with straps on both sides of the facemask. In all subjects, the facemask that offered the greater comfort while avoiding or minimizing leaks was used. The ECT consisted of 3 stages; a warm-up stage (70W for 10 minutes), an exercise stage (increasing power equivalent to an oxygen consumption that represented 60% of the difference between first ventilation threshold (VT1) and $VO_{2peak,}$ which was continuous until volitional fatigue) and a cool-down stage (power decreased to 50W for 10 minutes). The subjects were “blinded” to the lapsed time of each endurance exercise test in order to remove the possibilities for the subject to prepare for the end of “warm up” phase and also to eliminate the possibilities for the subjects to match the times of each test.

3.4 Global Gene Expression Changes – protocols and equipment

3.4.1 Blood Collection and Phlebotomy

Approximately 8mL of venous blood was sampled from an ante-cubital vein into an EDTA tube at three separate time-points over the two weeks. EDTA tubes ensure mixing of anticoagulant with blood to prevent clotting. Blood was only collected in the morning from n=18 individuals and obtained at baseline, 2 weeks post baseline (immediately post the last session in week 2) and 72 hours post training week 2. A total of 54 samples were collected.

These time-points were chosen based on previous findings concerning gene expression changes in response to exercise (Connolly et al., 2004; Zieker et al., 2005b; Burgomaster et al., 2007; Radom-Aizik et al., 2008) and in order to provide a broad observation period due to the novel nature of this study, which examined genome wide changes in a highly trained population.

3.4.2 Cell Isolation: Density Gradient Centrifugation

In order to separate mononuclear cells (lymphocytes and monocytes) from the other elements found in the blood, samples underwent centrifugation with Ficoll-Paque PLUS, which has a low viscosity and a density of 1.077g/mL and which consists of a mixture of the polysaccharide Ficoll and sodium diatrizoate (Boyum, 1968a, 1968b, 1976). Ficoll-Paque PLUS efficiently aggregates red blood cells, which increases the sedimentation of the red cells (see Figure 10). Granulocytes also rapidly sediment down the bottom of the Ficoll-Paque layer because immediate contact with the slightly hypertonic Ficoll-Paque PLUS medium increases their density. Subsequently, after the cells have been centrifuged, both the granulocytes and red blood cells are found at the bottom of the tube, underneath the Ficoll-Paque PLUS. Furthermore, lymphocytes, monocytes and platelets are not dense enough in makeup to penetrate into the Ficoll-Paque PLUS layer. For this reason, the cells are collected in a concentrated ring at the interface between the original blood sample and the Ficoll-Paque PLUS. This ring enables simple extraction of the lymphocyte and monocyte cell populations without compromising the yield of the small sample (Boyum, 1968a, 1968b, 1976).

After collection, anti-coagulated blood samples (4mL) were diluted with an equal volume of phosphate buffered saline (PBS). Ficoll-Paque PLUS (1.3mL) was aliquoted into six 15 mL Eppendorf tubes with 2.3mL of diluted blood layered over it. The 15 mL Eppendorf tubes were first centrifuged at a speed of $124g_{av}$, at 18 – 20 ^{0}C, for 10 minutes, and underwent a second round of centrifugation at $235g_{av}$, 18 – 20^{0}C for another 10 minutes. The white blood cells were then collected and placed into Eppendorf tubes and centrifuged at a speed of $3999g_{av}$, 4^{0}C, for 10 minutes. The harvested lymphocytes and monocytes are then washed and centrifuged in order to remove platelets and any contaminated Ficoll-Paque PLUS and plasma (Boyum, 1968a, 1968b, 1976).

Figure 10 – Separation of white blood cells from whole blood using Ficoll-Paque PLUS

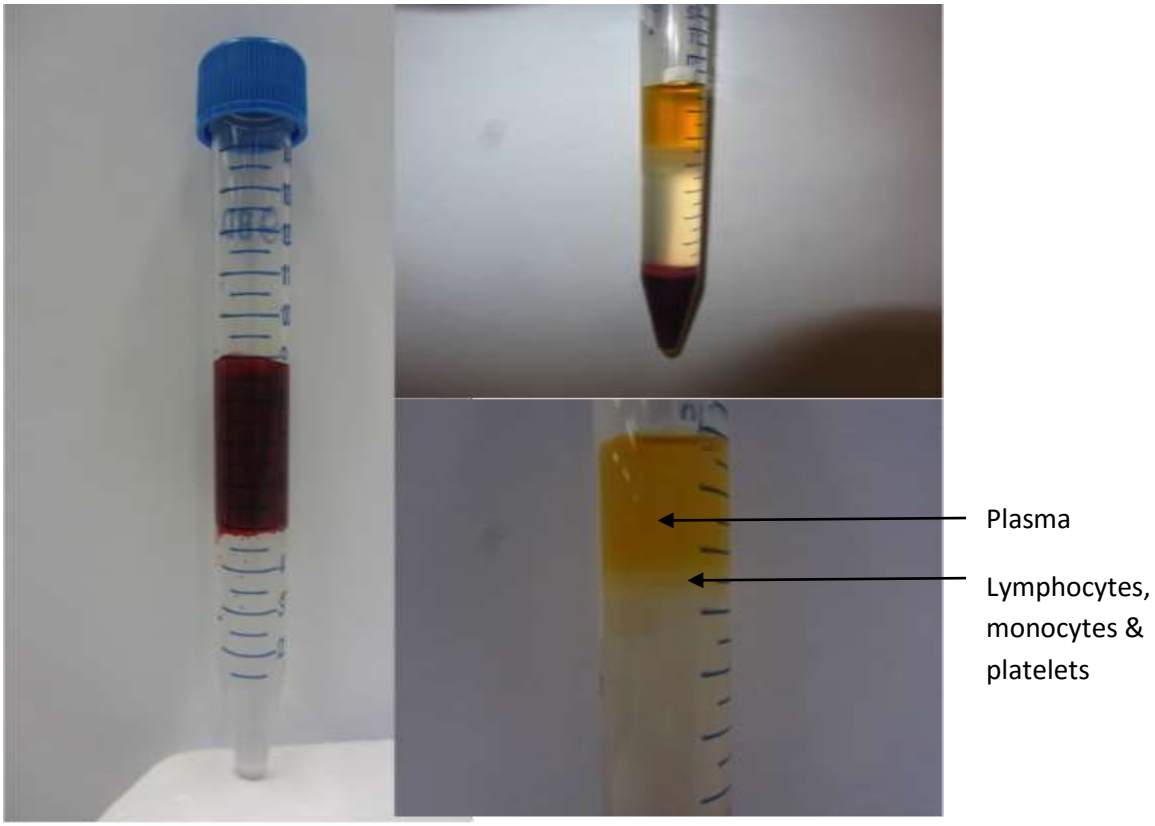


Fig10_Procedure for the isolation of lymphocytes and monocytes ("Ficoll-Paque PLUS for *in vitro* isolation of lymphocytes," 2001). Samples underwent centrifugation with Ficoll-Paque PLUS with granulocytes and red blood cells residing at the bottom of the tube, underneath the Ficoll-Paque PLUS. Collection of Lymphocytes, monocytes and platelets is undertaken from the concentrated ring appearing at the interface between the plasma (on top) and the Ficoll-Paque PLUS (at the bottom).

3.4.3 Total RNA Purification: Solid Phase Extraction

There are four key steps involved in the solid phase extraction procedure: cell lysis, nucleic acid absorption, washing and elution (Gjerde et al., 2009; Nicosia et al., 2010). The technology of RNA purification combines the selective binding properties of a silica-based membrane with the speed of microspin technology (Gjerde et al., 2009; Nicosia et al., 2010). A high-salt buffer allows up to 100µg of RNA longer than 200 bases to bind to the RNeasy silica membrane (Gjerde et al., 2009; Nicosia et al., 2010). The initial step in this process is to condition the column for sample absorption. This can be performed by using a buffer that has a particular pH in order to convert the surface on the solid into a particular chemical form (Gjerde et al., 2009; Nicosia et al., 2010).

The white blood cells harvested from the centrifugation step with Ficoll-Paque PLUS were first lysed and homogenised in Trizol. This treatment with Trizol immediately inactivates RNases to ensure an optimal integrity of RNA (Gjerde et al., 2009; Nicosia et al., 2010). Ethanol was then added, in order to provide appropriate binding conditions within the RNeasy silica based columns (Gjerde et al., 2009; Nicosia et al., 2010). Following this step, the lysed samples were processed according to the manufacturer's procedures. Briefly, lysis of cells was performed by passage through pipette tips several times and incubated for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. 0.2 mL chloroform was then added and gently mixed by hand for 15 s. Following 3 min incubation (room temperature), the sample was centrifuged at 12,000 x g for 15 min at 4°C. A colourless upper phase containing the RNA was collected gently and transferred into a new sterile tube containing an equal volume of 70% ethanol. Samples were vortexed for 30 s and placed within a RNA Spin column.

Total RNA samples obtained following Trizol treatment were applied to the RNeasy mini spin column. With both high pH and salt concentrations, the binding solution included in this kit enables nucleic acid molecules to attach to the column (Gjerde et al., 2009; Nicosia et al., 2010). Samples were subsequently treated with RNase free-DNase I for 15 minutes to remove all traces of residual DNA from columns prior to column washing and elution of purified RNA, as per manufacturer's instructions. In the washing step, other compounds such as proteins that may potentially contaminate the sample are removed using a washing

buffer (Gjerde et al., 2009; Nicosia et al., 2010). Tris EDTA (TE) buffer was then poured as a final step, known as the elution step, in order to release the desired nucleic acid from the column's attachment. The nucleic acid molecules were then collected in a purified state (Gjerde et al., 2009; Nicosia et al., 2010). The RNA was eluted into 100 µL of pure DNAse and RNAse free water and quality control was established on RNA samples. Finally, assessment of both RNA concentration and purity was undertaken using the NanoDrop 1000 spectrophotometer (Nanodrop 1000, Thermo Scientific). Reading ratio measurements of 260/280 and 260/230 ratios assessed RNA quality.

The step-by-step protocol for RNA extraction may be seen in Appendix E.

3.4.4 Reverse transcription to make cDNA

Total RNA was isolated from whole blood and treated to synthesise cDNA template to further undertake PCR experiments using SuperScript® III First-Strand Synthesis System kit: catalogue number 18080-051. The first phase of the RT-PCR process turns RNA into single-stranded cDNA products (Freeman et al., 1999; Bustin & Mueller, 2005). The enzyme responsible for this process is reverse transcriptase, which holds an RNA-dependent DNA polymerase activity. The enzyme recognises the RNA molecules by means of oligonucleotide primers which anneal to the RNA molecules and extend a DNA molecule by including units of deoxyribonucleotides in the forming DNA (Freeman et al., 1999). Primers in use can be either target gene-specific or non-specific (Freeman et al., 1999). When using target gene-specific primers, the RT reactions occur at elevated temperatures (Freeman et al., 1999). In regard to unwanted transcripts, this option will increase specificity and decrease background noise (Freeman et al., 1999). However, the use of gene-specific primers necessitates that a separate RT reaction be carried out for each gene of interest which can result in high inter-assay variability (Freeman et al., 1999). In contrast, separate target PCR reactions can be assayed from a cDNA synthesis "pool", which results from the use of non-specific primers (Freeman et al., 1999). Non-specific primers include random hexamers—which contain all possible nucleotide combinations of a 6-base oligonucleotide and which allow for binding at multiple origins along every RNA template—and poly-T oligonucleotides (Oligo-dT)—which consist solely of 16-25 deoxythymidine residues and which allow the annealing to the polyadenylated 3' (poly-A) tails found on most mRNA (Freeman et al., 1999). Interestingly, cDNA can be reversed transcribed with mature mRNA by using oligo-dT

primers (Bustin, 2002). This transformation of mRNA to cDNA is possible by polyadenylation through the usage of poly (A) polymerase (Bustin, 2002). In order to maximize the number of genes that can be assayed from small RNA yields, RT reactions were undertaken on the isolated RNA primed by non-specific random hexamers and oligo-dT primers (Freeman et al., 1999).

3.4.5 Genome-wide Microarray

Blood was collected from all subjects (n=18) and analysed at baseline, 2 weeks post baseline (immediately post the last session in week 2) and 72 hours post training week 2, a total of 54 samples. In order to qualify for microarray analysis, the samples had to pass quality control. Samples purity and integrity was assessed as having a spectrophotometer A260/280 ratio of >1.6 and an RNA integrity number (RIN number) of >7, respectively. Only 30 samples passed this assessment (G.1 n=6, & G.2 n=4) (Appendix G). HT12 Illumina full transcriptome Expression Arrays were interrogated to determine the expression levels of thousands of genes. These arrays were run to identify a set of genes that could be differentially expressed in response to 2 weeks of sprint interval training (SIT). More precisely, Illumina gene expression beadchips consist of oligonucleotides immobilized to beads held in microwells on the surface of an array substrate (Schulze & Downward, 2001; Korol, 2003). Data quality and reproducibility are supported in part by the high level bead-type redundancy on every array (Schulze & Downward, 2001; Korol, 2003). After randomly distributing the beads across the substrate surface, 29-mer address steps, BeadChips were scanned on a Hiscan^{TM}SQ, Iscan, or BeadArray^{TM} reader. The critical steps in this process included the selection and nature of the DNA sequences that were placed on the array, and the technique of fixing the sequences to the substrate. Sequences present on each bead underwent a hybridization-based procedure to map the array, identifying the location of each bead. This final process also validates the hybridization performance of every bead on every BeadChip, ensuring 100% quality control (Schulze & Downward, 2001; Korol, 2003). Illumina expression bead chip arrays are arranged in a multi-sample format for higher throughput and reduced sample-to-sample variability (Schulze & Downward, 2001; Korol, 2003). Sample cDNA was detected by hybridization to 50-mer probes on the BeadChip (Schulze & Downward, 2001; Korol, 2003). Microarray processing was carried out using the manufacturer's protocol (http://support.illumina.com/content/dam/illumina-support/documents/myillumina/3466b

f71-78bd-4842-8bfc-393a45d11874/wggex_ direct_hybridization_assay_guide_11322355_a .pdf).

After washing the labelled DNA, microarrays quantify gene expression by means of fluorescence intensity, which is captured by the scanners onto an image. The images are converted to numbers which are relatively reliable and stable (Schulze & Downward, 2001; Korol, 2003). Each technology generates different types of images and thus generates different quantities which have to be adequately operated to provide some kind of estimate of the gene expression (Schulze & Downward, 2001; Korol, 2003). This present study used the new HumanHT-12 v4.0 Expression BeadChip that supports highly efficient human whole-genome gene expression profiling studies. Its content provided a genome-wide transcriptional coverage of well-characterized genes, gene candidates and splice variants, with a significant portion targeting well-established sequences supported by peer-reviewed literature. Each array on this BeadChip targets more than 47,000 probes (Schulze & Downward, 2001; Korol, 2003).

3.4.6 Statistical analysis of Microarray

The Bioconductor software suite for bioinformatics (http://www.bioconductor.org/) was used to analyse the data with methods sourced from the Limma package. Bioconductor is an open-source and open-development software project for the analysis and comprehension of genomic data. It utilizes the R software package (http://www.r-project.org/), which is a language and environment for statistical computing and graphics. After processing the data with the Limma software, exploratory data analysis was undertaken. A box-plot displaying the distribution of intensities for each array was generated to investigate any differences in spread across the arrays.

The Lumi package provides unique functions for expression microarray processing. It includes a variance-stabilizing transformation (VST) algorithm that takes advantage of the technical replicates available on every Illumina microarray. The quality control of a LumiBatch object included a data summary (the mean and standard deviation, sample correlation, detectable probe ratio of each sample (microarray), divergent quality control plots, and the control probe information). The quality control plots included: a density plot,

a box plot pairwise scatter plot between microarrays or a pair scatter plot with smoothing, a pairwise MAplot between microarrays or an MAplot with smoothing and a density plot of coefficient of variance.

"Normalization" reduces the amount of variability in the data that is due to non-biological sources, so reducing variability of non-differentially expressed genes and this is achieved using a quantile normalization procedure (Quackenbush, 2002). The normalization process was as follows: i) Data is log 2 transformed given the inherent exponential nature of all hybridization results; ii) A normalization factor N is calculated using the relative sums technique for each hybridization pair (control/intervention); iii) Then Z scores are calculated based on a 95% confidence interval. Due to these stringent procedures, the resulting normalized data set has a much more consistent distribution of intensities with less variability from non-biological sources. For this study, raw data was normalised and differential gene expressions across the human white blood cells were calculated and expressed as Z scores. All z scores for each gene were further processed and put through a linear regression analysis. Slope values obtained from the linear regression plot and specific to one candidate gene, were used to generate an "M" value. Each individual gene was evaluated through its Z scores values using this M value (positive to negative values). The M value for a corresponding gene reflected the trend of gene expression. Finally, p-values describing the significance of differentially expressed genes were adjusted for multiple hypothesis testing using the Benjamin-Hochberg method (Benjamin & Hochberg, 1995).

3.4.7 Reverse Transcription- PCR

Quantification of steady-state mRNA levels by reverse transcription polymerase chain reaction (RT-PCR) is typically undertaken after microarray analysis in order to validate the gene expression analysis and candidate findings of the microarray. The technique is highly sensitive, permitting analysis of gene expression from very small amounts of RNA (Freeman et al., 1999; Bustin & Mueller, 2005). Furthermore, RT-PCR can be conducted on a large number of samples and/or many different genes in the same experiment (Freeman et al., 1999; Bustin & Mueller, 2005). As a result, investigators obtain a measure of flexibility that is unavailable in more traditional approaches of expression level validation (i.e., Northern blot, hybridization protection assays, dot plot and in situ hybridization) (Freeman et al.,

1999). However, the RT-PCR technique requires some care, as the amplification technology is prone to amplifying errors associated with the experiment (Freeman et al., 1999). Consequently, variability in the results may prevent accuracy and reliability of the amplification data (Freeman et al., 1999). This problem can be solved by thoughtful experimental design and careful validation of the technique (Freeman et al., 1999).

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction or PCR is a method of amplifying a specific target sequence through repeated cycles of temperature management, which assists enzymatic replication of the target amplicon (Arya et al., 2005; Bustin & Mueller, 2005). This process utilizes the capacity of the thermostable, DNA polymerase (Taq), from *Thermus aquaticus*, to synthesize DNA strands (Bustin & Mueller, 2005). However, DNA polymerase can only add free nucleotides to a pre-existing 3'-OH group, demanding that oligonucleotide primers that exhibit unique complementarity to the target amplicon be base paired to the template sequences (Hartl, 2011). Each of the reaction cycles consist of a three step process:

1) Initiation of the reaction is to increase the heat to 94^{o}C that results in denaturation of all present ds-DNA (Bustin, 2002).

2) The reaction is then cooled to the range of 40 – 60^{o}C, which is the specific annealing temperature of the present primers (Bustin, 2002). This allows the primers to hybridize to their complementary sequence on opposite strands of the target amplicon (Bustin, 2002).

3) Finally, in order to allow for elongation of the template strands by DNA polymerase, the cycle is heated to 72^{o}C (Bustin, 2002).

There are three distinct reaction phases of product amplification that the PCR reaction undergoes in its progression: the exponential phase, the linear phase and the plateau phase. Amplicon production in the exponential phase is very efficient (Bustin, 2002). Following each successive cycle, the amount of DNA synthesised is theoretically doubled. However, this efficiency slows down in the linear phase of amplification due to a decrease in the

critical concentration of one or more reaction components (Bustin, 2002). For this reason, changes in amplified product are observed on an arithmetic rather than a logarithmic scale (Bustin, 2002). When the PCR reaches its plateau phase, there is little or no rise in PCR product due to the increasingly limited reaction components (Bustin, 2002). Furthermore, only during the exponential phase of PCR amplification is the amount of amplified target directly proportional to its original concentration in the starting template (Bustin, 2002).

Design of PCR primers specific to particular genes:

Gene DNA sequences were obtained using Ensembl (http://www.ensembl.org/). Primers for the internal control 18s and the other genes investigated were designed using primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) with the following primer sequences attributed to the following genes:

18s: forward primer 5'CTTAGAGGGACAAGTGGCG3' and reverse primer 5'GGACGTCTAAAGGGCATCACA3'

Gene Il6R: forward primer 5'CACGACTCTGGAAACTATTCATGCTA3' and reverse primer 5'GGGAACATCCACCAGCAAGT3'

Gene IL16: forward primer 5'TCTGCAGCCAGTGATGTTTC3' and reverse primer 5'GAGGCTTGTCTCCGTGTAGG3'

Gene CD69: forward primer 5'TGCCATCAGACAGCCATGTT3' and reverse primer 5'TGACCACTTCCATGGGTGAC3'

Of note, these primers are tabulated in Table 11.

PCR amplification:

All these primers were used in PCR amplifications using the sample-generated cDNA (template starting material diluted 25x into DNase/RNase free pure water) from group 1 and group 2 cohorts. The gene 18s was used as the internal housekeeping gene for Q-PCR data analysis purposes (see below). PCR runs were undertaken in triplicate for each gene on each cDNA template using a reaction cocktail (final volume of 25µL) that would include the

addition of 12.5 uL of Biorad of IQ™Sybr Green® Supermix (cat nb 170-8882), 1 uL of 5 mM of each forward and reverse primer solution, 2 uL of cDNA and finally overlay with pure water to 25uL final reaction volume. PCR runs were carried out in individual Qiagen/Corbett 0.1 mL strip tubes (Qiagen Type 4 strip Tube & Caps, Bulk, 250/pk cat nb: 981103) previously placed on a minus 20°C pre-cooled metal tube carrier in the following order: cDNA first followed by the resulting PCR mix. Tubes were closed off and manually handled to allow the whole PCR solution to rest at the bottom of the tube. Real time detection of PCR products was performed using the Corbett Research Rotor-Gene 3000 (Qiagen-Corbett, Corbett Rotor-Gene 6000). All PCR tubes were placed in the 72 well Rotor within the real-time PCR machine. The following cycling conditions were inputted in the Rotor Gene software as follows: Cycle 1, 94°C 12 min (x1), Cycle 2, 94°C 30 s, 59°C 30 s, 72°C 30 s (x45). A post-PCR amplification protocol was pre-set before the run to obtain melt curve representations (ramping from 50° to 99°C with 1°C ramping every 5 seconds).

PCR optimization: Optimisation of PCR was not necessary as each primer set was designed to anneal at 59°C. In addition, sufficient elongation time was used to cover the whole length of the amplicons. Finally, the protocol enabled a "hotstart" activation of the enzyme contained in the Biorad IQ™Sybr Green® Supermix with an initial cycle of 12 minutes at 94 °C being performed.

Amplicon Detection

Real-time RT-PCR measures the progress of amplification through the monitoring of changes in fluorescence within the PCR reaction (Pfaffl, 2004). To achieve this, SYBR Green which is a non-sequence specific fluorescent intercalating double stranded (dsDNA) binding agent, is added to the reaction mix (Pfaffl, 2004). SYBR Green displays an extremely low intrinsic fluorescence due to its excitation maximum of 497nm and emission maximum of 520nm (Skeidsvoll & Ueland, 1995). Conversely, when intercalated into dsDNA, its fluorescence quantum yield is increased by up to 1000 fold (Skeidsvoll & Ueland, 1995). Consequently, the intensity of the fluorescent signal is dependent on the quantity of dsDNA present in the reaction (Skeidsvoll & Ueland, 1995). The level of fluorescence is measured only when the quantity of dsDNA reaches its maximum level, which is at the end of the elongation phase of each PCR cycle (Skeidsvoll & Ueland, 1995). However, the dye binds to all dsDNA formed during the PCR reaction and is therefore not specific to the target amplicon (Bustin, 2002;

Bustin & Mueller, 2005). This allows the presence of non-specific products and "primer-dimers" to generate artefact within the fluorescent signal, which is a limitation to the use of SYBR Green (Saunders, 2004; Bustin & Mueller, 2005). Therefore, melt curve analysis is used in order to monitor the specificity of this reaction (Saunders, 2004; Bustin & Mueller, 2005). As the temperature of the reaction in the end of the PCR run gradually rises, the fluorescence is constantly measured (Bustin, 2002; Saunders, 2004; Bustin & Mueller, 2005). The temperature rises from a low temperature where all amplified products are stable to a point at which all dsDNA present has become fully dissociated (Saunders, 2004). By plotting the negative first derivative of the change in fluorescence as a function of temperature (-dF/dT) against temperature, melt curves are successively generated (see Figure 11) (Saunders, 2004; Bustin & Mueller, 2005). The identification of characteristic peaks at the melting temperature (Tm) of the amplicon (which corresponds to the point at which 50% of target DNA is denatured) will distinguish it from amplification artefacts that melt at lower temperatures and exhibit broader peaks (Saunders, 2004; Bustin & Mueller, 2005).

Figure 11 - RT-PCR Melt Curve

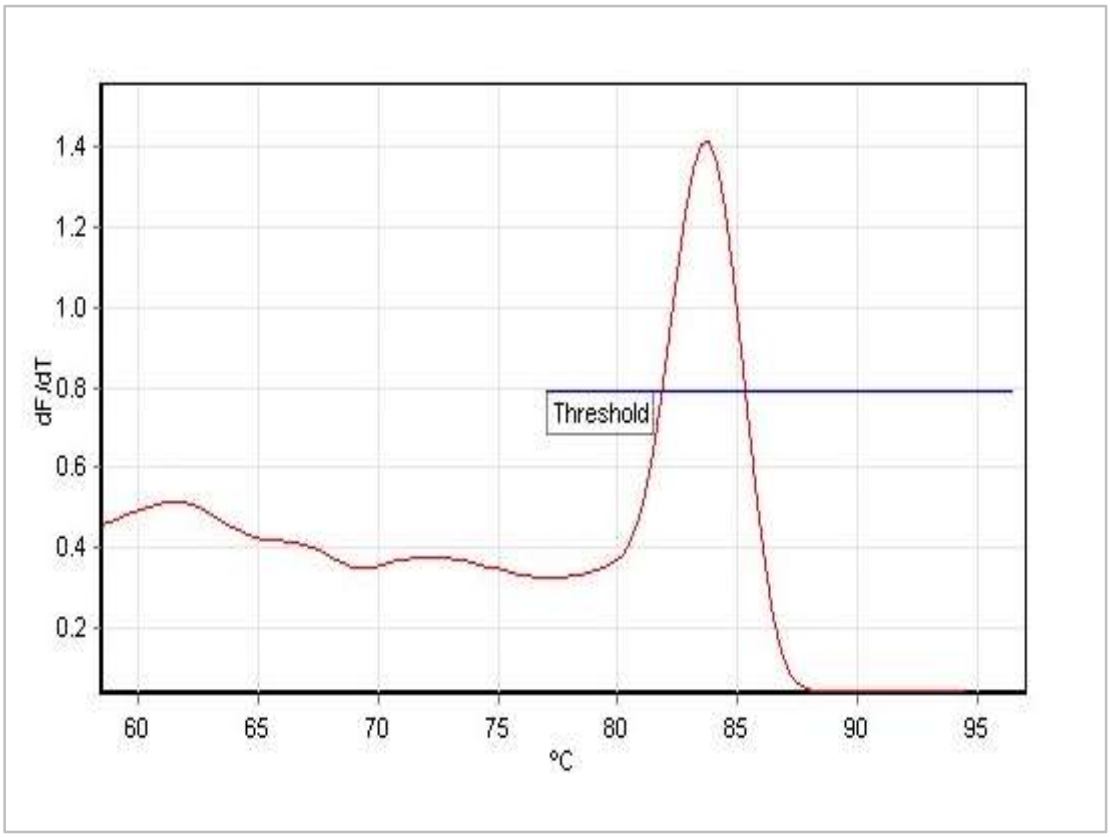


Fig11_Melt curve from study results displaying the negative first derivative of the change in fluorescence as a function of temperature (-dF/dT) against temperature (^{0}C).

RT-PCR Data Analysis

During PCR data collection, fluorescence emissions are measured in order to generate amplification plots as seen in Figure 12. During the initial PCR cycles, a baseline reading is recorded where a fluorescent signal is accumulated beneath the detection capabilities of the instrument (Arya et al., 2005). Quantification in real-time RT-PCR is achieved through the measurement of the number of cycles required for the fluorescent signal to cross a threshold level (Arya et al., 2005).

Figure 12 - RT-PCR Cycle threshold

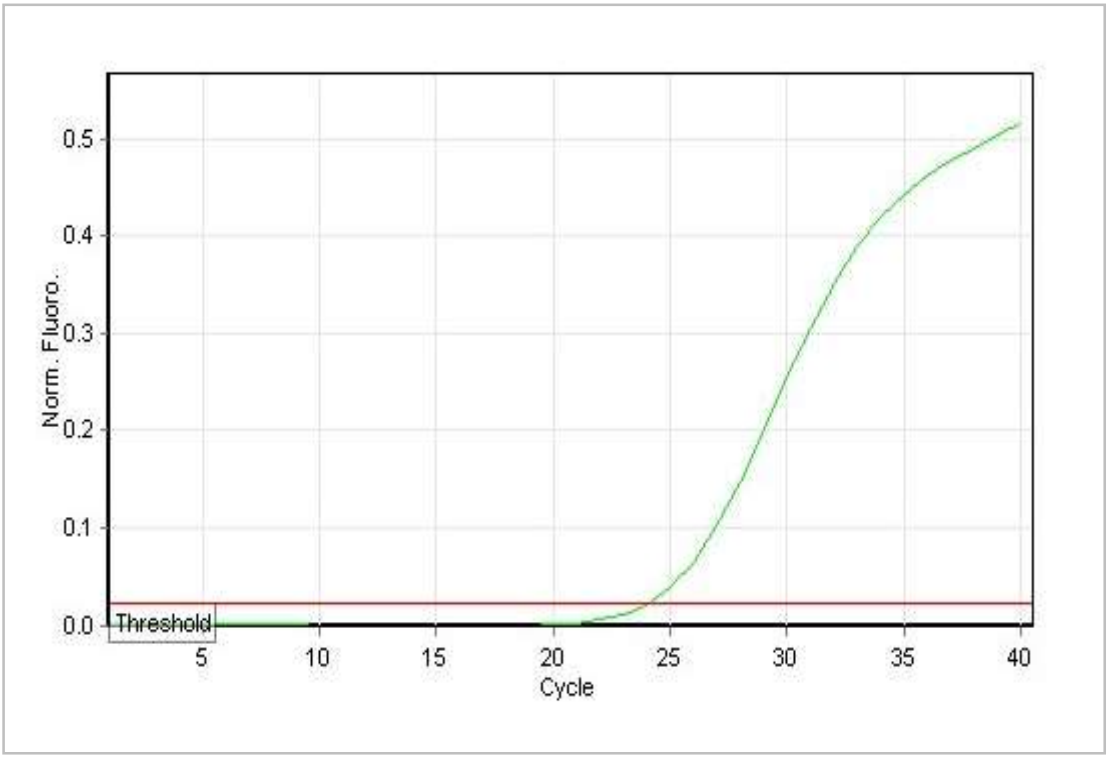


Fig12_Typical RT-PCR amplification plot of the study results: display of fluorescence emissions against cycle threshold.

In order to produce accurate and reproducible data, this arbitrary threshold must be set at a value at least three standard deviations above the baseline noise level and occur in the exponential phase of target amplification (Arya et al., 2005). The cycle number at which a fluorescent signal is detected above this chosen threshold value is defined as the “cycle threshold” or Ct (Arya et al., 2005). The Ct of a sample is inversely proportional to the

concentration of target sequence in the starting template (Arya et al., 2005). Thus, the more target copies present at the beginning of a reaction, the fewer cycles of amplification that are required to generate the number of amplicons which can be detected reliably (Arya et al., 2005).

Relative Quantification

In order to determine approximate changes in the steady state expression of a gene across multiple samples relative to the expression of an internal control RNA, relative quantification uses the comparative threshold method ($2^{-\Delta\Delta Ct}$ method) (Arya et al., 2005). The method compares the Ct values from target RNA to those from one or more endogenous reference or 'housekeeping' genes with the results expressed as ratios of the target specific signal to that of the endogenous reference (Arya et al., 2005). In order to calculate the expression of "experimental" target samples in relation to "reference" control samples such as non-treated or placebo samples, mathematical equations are used (Arya et al., 2005). The amount of target RNA in an experimental sample, normalized to an endogenous housekeeping gene and relative to the normalized control sample is thus given by equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (experimental sample) – ΔCt (control sample), and ΔCt = Ct (target gene) – Ct (reference gene) (Arya et al., 2005).

Data Normalization

Due to minor variances in the quantity and quality of template RNA or disparity in the efficiency of cDNA synthesis and PCR amplification, the accuracy of calculated expression outcomes in real-time RT-PCR experiments are significantly affected (Pfaffl, 2004). In order to control for the significant error presented by these parameters, it is critical to apply a normalization strategy (Pfaffl, 2004). To improve the reliability of any relative real-time RT-PCR experiments and to correct for sample to sample variation in RT-PCR efficiency and errors in sample quantification, the use of a housekeeping gene can be used to serve as an endogenous reference control in the assay (Pfaffl, 2004). The most commonly used housekeeping genes include β-actin – a cytoskeletal protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) – a glycolytic enzyme and 18s or 28s – ribosomal RNAs (Pfaffl, 2004; Arya et al., 2005). The expression levels of all of these genes should stay rather constant under different experimental conditions, as they are necessary for basic cell survival.

Furthermore, the genes should theoretically be expressed at a constant level among different cell types at all stages of development (Pfaffl, 2004; Arya et al., 2005). However, as all are regulated in some capacity and none are constitutively expressed under all conditions in all cell types, no single gene is capable of meeting the criteria required of a universal reference gene (Pfaffl, 2004; Arya et al., 2005).

Therefore, the expression stability of selected housekeeping genes must be experimentally validated for the cell population under investigation as the reliability of the expression data depends on the choice of the most relevant housekeeping gene for the cells of interest (Bustin & Mueller, 2005). Moreover, as even small differences directly affect the accuracy of any calculated expression result, it is vital that the amplification efficiencies of target and reference samples are similar (Bustin & Mueller, 2005; Pfaffl, 2004). A variance in amplification efficiency of 3% between target and reference gene amplicons is reported to produce error in the calculated expression ratio of 47% when target amplification productivity exceeds reference amplification efficiency (Pfaffl, 2004). Furthermore, a production error of 209% is found when reference amplification efficiency exceeds target amplification efficiency after 25 cycles of the PCR run (Pfaffl, 2004). It is therefore critical that selected primers are validated to achieve a directly equivalent level of efficiency for the target and housekeeping gene amplicons under investigation (Pfaffl, 2004).

Chapter 4 - Physiological Changes caused by Sprint Interval Training Intervention

4.1. Introduction

High intensity interval training (HIT) is a well-known time efficient training method for improving performance in sedentary and recreationally trained individuals, as well as athletes. Additionally, the physiological adaptations in response to HIT are also reasonably well understood. Significant improvements in physiological markers and improvements in endurance performance are obvious after sub-maximal endurance training in untrained and active individuals. Furthermore, it is known that in trained individuals an additional increase in low intensity training volume does not necessarily further increase endurance performance or any associated physiological variables such as VO_{2peak} or oxidative enzyme activity (Laursen et al., 2004; Laursen et al., 2005). However, further increases in physiological and molecular changes can be made with HIT (Laursen et al., 2005).

This type of training has been shown to increase the concentration of energetic substrates and the activity of anaerobic metabolism related enzymes (Laursen & Jenkins, 2002) through an up regulation in the expression of their related genes, and has been successfully used as an alternative method when promoting endurance performance (Ross & Leveritt, 2001; Hawley, 2002b; Burgomaster et al., 2008). Furthermore, HIT program optimisation research in cyclists has shown that sprint interval training (SIT) may be equally as effective as more traditional HIT programs for improving endurance performance (Laursen et al., 2002). SIT performed across weeks or months have been shown in several studies to induce profound changes in skeletal muscle metabolism at physiological, biochemical and molecular levels (Parra et al., 2000; Hawley, 2002b; Burgomaster et al., 2005; Burgomaster et al., 2008). Some of the changes seen after SIT include an increased fractional muscle O_2 capacity and extraction, faster VO_2 kinetics, an increase in motor unit activation, increased exercise plasma lactate levels and an increased capacity for glucose and fatty acid oxidation (Barnett et al., 2004; Creer et al., 2004; Burgomaster et al., 2005; Burgomaster et al., 2006; Gibala et al., 2006; Burgomaster et al., 2007; Burgomaster et al., 2008; Bailey et al., 2009;

Gibala et al., 2009). Additionally, it has been shown that as little as eighteen repetitions per week for two weeks reduces metabolic risk factors in a young and sedentary population (Babraj et al., 2009). Whether as little as eighteen repetitions of SIT over two weeks can improve endurance performance has not yet been investigated. Of note, preferable optimal session frequency aiming at assessing endurance performance requires further research.

Furthermore, different responses in the biological system following SIT seems to occur in the individual athlete (Buchheit & Laursen, 2013). As an example, muscle performance appears to recover faster than muscle glycogen repletion (Docherty & Sporer, 2000; Krustrup et al., 2004; Binnie et al., 2013). Nevertheless, the current training recommendation for moderately trained athletes is to allow 48h recovery between HIT sessions to allow the majority of athletes to perform and train maximally (James et al., 2002; Hautala et al., 2009; Buchheit & Laursen, 2013). However, apart from a study by Dalleck et al. (2010) who found that by performing 6-8 SIT two times per week for six weeks instead of one time per week increased lactate thresholds favourably, no study has investigated whether SIT frequency is a factor for changes in performance. Due to a lack of accurate measuring techniques for recovery of the overall metabolic and neuromuscular systems, assessment of cardiac autonomic function (ANS) via heart rate variability (HRV) has appeared as an encouraging alternative (Buchheit & Laursen, 2013). Actually, HRV has been suggested as a tool to individualize the programming of athletes' HIT and SIT sessions (James et al., 2002; Hautala et al., 2009; Buchheit & Laursen, 2013). However, limitations of this method must be acknowledged in relation to tracking some biological variables such as glycogen stores and neuromuscular function (Hautala et al., 2009; Buchheit & Laursen, 2013).

Based on the current research, this study will investigate whether eighteen repetitions over two weeks will increase endurance capacity in trained cyclists. Furthermore, this study aims to investigate if there is a difference in endurance performance when these eighteen repetitions are delivered in six versus two sessions over the two weeks.

4.2. Methods

Trained cyclists aged 18-35 years old (n=26) were randomly assigned to either group 1(n=9) or group 2 (n=9) and performed performance tests before and after the 2-wk sprint interval training intervention. A control group (n=8) carried out the performance tests 2-wks apart from each other without doing any training intervention. All subjects had a minimum of three sessions on an ergometer per week. After a routine medical screening, all the subjects were informed about the procedures and protocols used in the study. Bond University Human Research Ethics Committee (BUHREC) approved this project; ethics number RO1063.

Dietary and exercise control: In an attempt to minimize any potential diet and training-induced variability, all subjects were instructed to keep their diet and training regime similar throughout their involvement in the project. The subjects were instructed to record all dietary intake as well as training sessions throughout the whole project and compliance was assessed by performing a dietary and training analysis on the individual food and training records maintained by the subjects. This was quantified through a diary designed particularly for this project, where each subject recorded in detail their dietary intake and training sessions throughout their involvement in this study (Appendix F).

Testing: A maximal oxygen consumption test (VO_2max), an Endurance capacity test (ECT) and a Wingate test were performed 3-days prior and 3-days post training intervention.

Training intervention: Each training session consisted of repeated 30 s all out efforts on an electronically braked cycle ergometer (Lode, The Netherlands). During the 4 min recovery between each 30 s test, the subjects remained on the ergometer and continued cycling at a low cadence. G1 group came in for training three times per week on Monday's, Wednesday's and Friday's for two weeks. G2 group attended training once per week on Wednesdays for two weeks.

Statistical analysis: Results are reported as mean ± SD. The duration of the constant-work rate exercises was defined as the endurance time. Endurance time to exhaustion was assessed using a paired-t test. The values for all other variables were assessed using a two-way between-subject factor repeated measure ANOVA. Post-hoc comparisons were

identified using Bonferroni-corrected t tests. A statistical level of significance of $p < 0.05$ was used for all analyses.

4.2.1 Optimal ECT apparatus sub-study: Mouthpiece or facemask?

Open Circuit Spirometry is widely used to measure the volume of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) in established exercise testing protocols. This method draws upon the Fick equation and subsequent Haldane transformations to calculate VO_2 and VCO_2. Integral to these calculations is a measure of airflow, which is used to determine a subject's rate of ventilation (V_E). Errors associated with measuring V_E can have significant consequences upon the calculation of VO_2 and VCO_2 and the resulting interpretation from those calculations.

Traditionally, a mouthpiece with nose clip have been used to capture the expired ventilation during maximal and sub-maximal exercise testing, often with some discomfort to the subject, particularly at high rates of ventilation. Bloch et al. (1995) suggested that in healthy men, undergoing maximum exercise testing using a mouthpiece and nose clip may result in a premature end to an incremental exercise test before the benchmarks for maximum oxygen consumption have been satisfied. To mitigate the discomfort and therefore reduce the potential for early termination of an incremental exercise test, a facemask has been used as a substitute method to capture the expired ventilation (Saey et al., 2006). However, while the alternate use of a facemask may remove or lessen the discomfort experienced by the individual, there may be leakage of expired air at the interface between the facemask and the skin of the face (Segal, 1987). Such leakages may increase with high rates of V_E that occur with athletes during an incremental exercise test or constant power test to exhaustion. Thus, Saey et al. (2006) investigated the difference in metabolic output when exercise testing was performed using a mouthpiece and nose clip compared with the use of a facemask and found that at ventilation rates of less than 50 $L.min^{-1}$, the mouth piece and nose clip resulted in significantly higher V_E, VO_2 and VCO_2 in patients with chronic obstructive pulmonary disease (COPD). However, no similar research has been conducted investigating the same equipment at higher ventilation rates in athletes.

Constant power exercise tests are increasingly used to predict performance of cyclists and with COPD patients to evaluate the efficacy of treatment. Given the increasing preference for use of a face mask over the mouth piece and nose clip by trained individuals during performance testing, it is important to know if there are significant differences in the measured V_E and subsequent calculations of VO_2 and VCO_2 between the two methods at higher rates of ventilation [70 $L \cdot min^{-1}$ to >100 $L \cdot min^{-1}$] typically seen in a constant power exercise test. Therefore, a sub-study was undertaken to compare the use of a mouthpiece/nose clip (MP) and facemask (FM) during a constant power cycle exercise test to exhaustion in active cyclists, measuring time to exhaustion, heart rate, measured rate of expired ventilation and the subsequent calculated oxygen consumption, carbon dioxide production and respiratory exchange ratio.

4.2.2 Optimal ECT apparatus sub-study methods

Ten healthy males aged 26.7 ± 6.3 years (see Table 8) were recruited to take part in the study. After a routine medical screening, all subjects were informed about the intended protocols and the associated risks. Subjects were required to give their written consent. Bond University Human Research Ethics Committee (BUHREC) reviewed and approved all protocols and procedures. All subjects completed a familiarization session with the intended protocols, procedures and equipment, including mouthpiece/nose clip and facemask. Small or medium facemasks were selected to ensure maximum contact with the skin of the face (Hans Rudolf 7600 Series V2 full face CPAP masks with headgear). The respective dead space for small or medium facemasks was 110mL and 136mL. The Medgraphics, medium adult mouthpiece with a built-in saliva trap was used for all subjects. Height and body mass were measured according to standard procedures. Pulmonary function was assessed using a calibrated spirometry system (Medical Graphics Corporation, USA). Supine and standing 12-lead ECG (Cardio Perfect, Welch Allyn Inc., USA) and blood pressure were measured at rest.

Table 8 - Characteristics for participating subjects (N=10)

Characteristic	Mean	Median	± SD
Age (yr.)	26.7	25.5	± 6.3
Height (cm)	179.0	180.7	± 7.9
Weight (kg)	78.9	78.3	± 7.0
HR ($b \cdot min^{-1}$) Supine	60.6	63.5	± 12.3
HR ($b \cdot min^{-1}$) Standing	69.2	70.6	± 13.5
FVC (L)	5.35	5.2	± 0.4
FVC % Predicted	93.52	94.1	± 8.1
FEV_1 (L)	4.27	4.29	± 0.3
FEV_1 % Predicted	91.27	91.13	± 11.1
FEV_1/FVC %	79.74	77.37	± 5.5
PEFR (FEF max) ($L \cdot s^{-1}$)	9.52	9.20	± 1.60
PEFR % Predicted	95.58	93.3	± 9.3
VO_2 ($L.min^{-1}$)	3.12	3.09	± 0.6
V_E BTPS ($L.min^{-1}$)	137.4	131.3	± 27.9
HR_{max} ($b \cdot min^{-1}$)	179	178	± 10.9

All subjects performed an incremental exercise test to volitional fatigue on an electronically braked cycle ergometer (Excalibur Sport: V2 Lode, Groningen, The Netherlands). The incremental exercise test included three stages: (I) warm up at 50W for 5 minutes, (II) an exercise stage starting at 50W with increasing power of 10W every 20 seconds until volitional fatigue or clinically significant signs or symptoms precluded further exercise and (III) cool down at 50W for 5 minutes followed by another 5 minutes of passive sitting. Subjects were fitted with a 5-lead ECG (Mortara Instrument Inc., USA) to monitor heart rate and rhythm throughout each exercise session. Brachial artery blood pressure was measured by auscultation during the early phases of the incremental exercise test. Carbon dioxide output (VCO_2), oxygen uptake (VO_2), and expired minute ventilation standardised to body temperature and pressure saturated (V_E BTPS) were measured breath-by-breath and then averaged every 30 seconds using open-circuit spirometry (Medical Graphics Corporation, St

Paul, USA). The gas analyzers and pneumotachograph were calibrated before and after each exercise test with adjustments to all calculations for any drift/offset during the course of the exercise test. The average of the two previous highest consecutive 30-second values measured from the point of volitional fatigue were used to determine maximum/peak gas exchange (VO_2, VCO_2) values. The gas exchange threshold (Tge) was determined using the simplified V-slope method (Schneider et al., 1993).

Each subject cycled to exhaustion on an electronically braked cycle ergometer (Excalibur Sport V2, Lode, Groningen, The Netherlands) at a power equivalent to an oxygen consumption that represented sixty percent of the difference between the oxygen consumption at Tge and at VO_{2peak} (Δ60% gas exchange threshold and VO_{2peak},) as modified by DiMenna et al. (2009). Each ECT started with a 10 min warm up at 70 W before the power was increased to match each subject's pre-determined exercise intensity. All ECTs were performed without any verbal or physiological feedback. The ECT was terminated when the pedal cadence fell below 40 rpm and the ECT duration was recorded. Expired ventilation was measured throughout the ECT and used to calculate minute ventilation rate (BTPS $L.min^{-1}$), oxygen uptake, carbon dioxide production, and respiratory exchange ratio which were averaged over two x 30 seconds collection periods. One ECT was performed using a mouthpiece (Medgraphics, medium adult mouthpiece with a built in saliva trap,) and a nose clip, and the other ECT was performed with a facemask [Hans-Rudolph 7600 series V2 full face CPAP mask with headgear].

Results are reported as mean ± SD. Exercise data collected during the facemask trial and mouthpiece/noseclip trial was compared using a two-way analysis of variance with repeated measures (time). Pairwise comparisons were performed when appropriate, using Bonferroni adjustments. Bland and Altman analysis (Bland & Altman, 1999) was used to investigate the agreement between sampling methods for key pulmonary gas-exchange measurements collected during submaximal exercise. Statistical significance was determined at an alpha level of $p < 0.05$.

4.3. Results

4.3.1 Optimal ECT apparatus sub-study _Results

The physiological responses throughout each ECT with mouthpiece/nose clip and facemask are presented in Figure 13. Although there were significant increases in the physiological responses over time, there were no significant differences between the mouthpiece/nose clip and facemask at any time-point for any measured or derived physiological response at low intensity or high intensity exercise.

Figure 13 - Physiological responses throughout each ECT

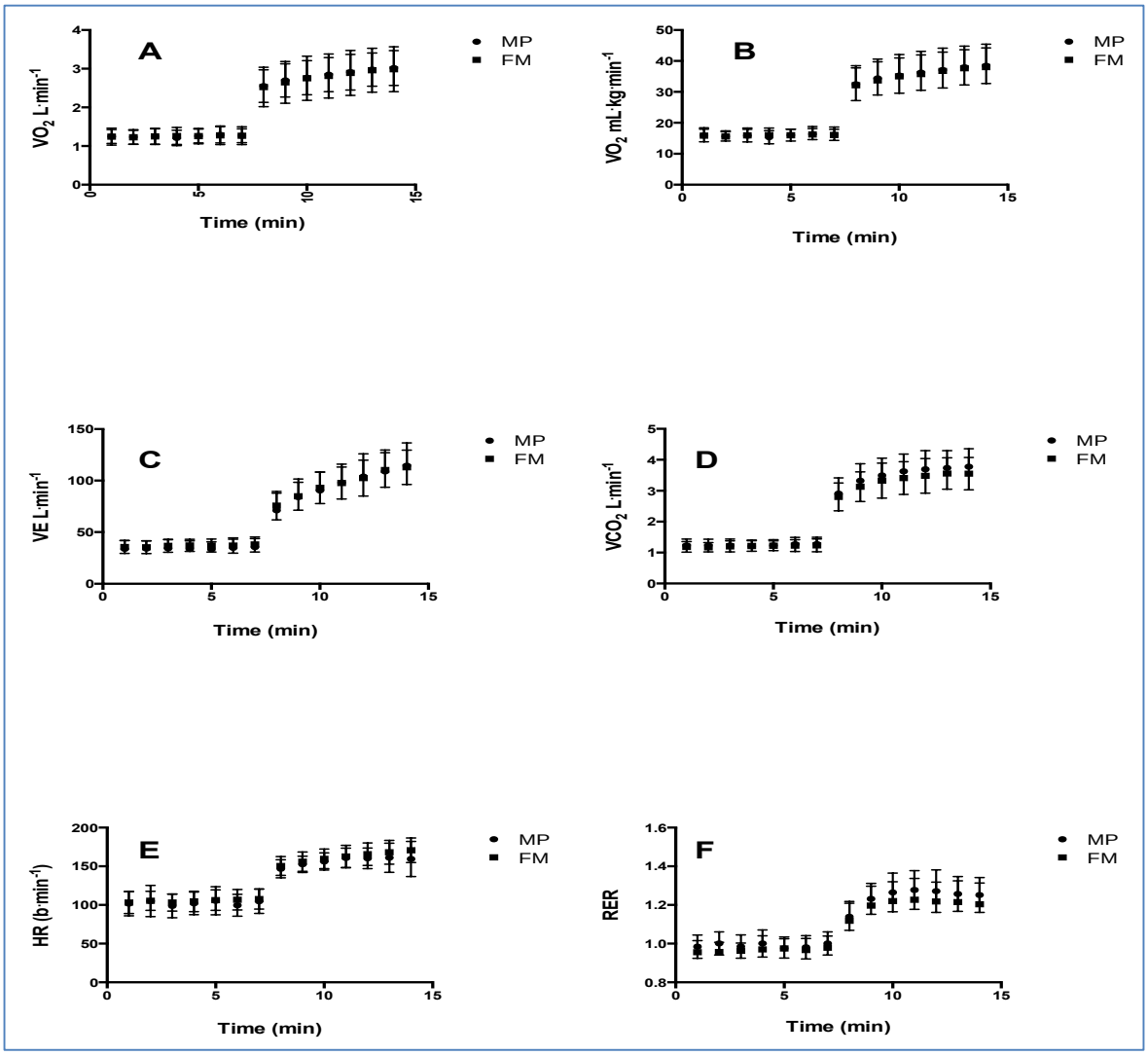


Fig13_ VO_2 $L \cdot min^{-1}$ (Panel A), VO_2 $ml \cdot kg^{-1} \cdot min^{-1}$ (Panel B), VE $L \cdot min^{-1}$ (Panel C), VCO_2 $L \cdot min^{-1}$ (Panel D), HR $b \cdot min^{-1}$ (Panel E) & RER (Panel F) response to exercise when using mouthpiece (closed dots) and facemask (open dots). The comparisons were done at the same absolute time at low intensity (70W) and high intensity (Δ60% of AT and VO_2max).

The physiological responses at the end of the ECT using the mouthpiece/nose clip and facemask are presented in Table 9. There were no significant differences in any physiological response between the mouth piece/nose clip and facemask at the end of the ECT. None of the physiological responses at VO_{2peak} and at the termination of the ECT with the mouth piece/nose clip and facemask were significantly different (Table 9).

Table 9 – ECT study results

Physiological Response	**Mouthpiece (N=10)**	**Facemask (N=10)**	**Significance**
	Mean ±SD	**Mean ±SD**	**p-value**
Endurance time (min)	14.08 ± 4.79	14.64 ± 5.06	p = 0.309
Heart rate ($b \cdot min^{-1}$)	170 ± 21	178± 12	p = 0.254
V_E ($L \cdot min^{-1}$) at BTPS	61.17 ± 33.6	62.96 ± 25.3	p = 0.163
Speed ($rev \cdot min^{-1}$)	73 ± 16	73 ± 11	p = 0.631
RER	1.22 ± 0.09	1.20 ± 0.11	p = 0.154
VO_2 ($L \cdot min^{-1}$)	3.08 ± 0.44	3.09 ± 0.66	p = 0.740
VCO_2 ($L \cdot min^{-1}$)	3.58 ± 0.75	3.52 ± 0.51	p = 0.773
* V_E, Ventilation; RER, Respiratory Exchange Ratio			

As shown in the Bland-Altman plot, determination of the differences in VO_2 (expressed in $L \cdot min^{-1}$) with the usage of the mouthpiece/nose clip and facemask are illustrated in Figure 14. Oxygen uptake collected using a mouthpiece/nose clip and facemask demonstrated strong agreement; scatter ($L \cdot min^{-1}$) did not display systematic trends. During moderate-intensity cycling (Figure 13), VO_2 collected via mouthpiece/nose clip was 0.01 ± 0.12 $L \cdot min^{-1}$ (i.e., 0.9 ± 9.5%) higher when compared with facemask. The bias for VO_2 measured during severe-intensity cycling was 0.02 ± 0.20 $L \cdot min^{-1}$ (i.e., 1.8 ± 8.5%) when compared with facemask. While wider limits of agreement were observed between the two methods at moderate-intensity, when differences in VO_2 were expressed relative to the average VO_2, very strong agreement was observed at higher amplitudes (e.g., error for all data > 2.25 $L \cdot min^{-1}$ was -1.9 ± 3.5 %).

Figure 14 - Differences in VO_2 L.min^{-1} vs. mean value VO_2 L·min^{-1}

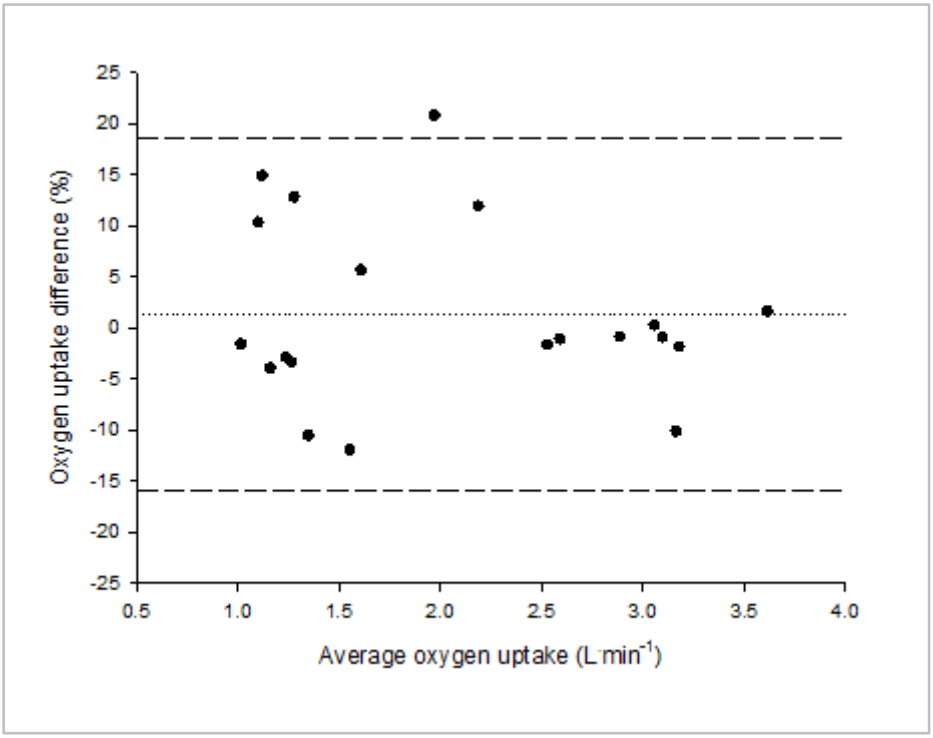


Fig 14_Individual level of agreement between VO_2 L·min^{-1} determined with the mouthpiece and the facemask, assessed according to the Bland-Altman approach. The difference in the VO_2 L·min^{-1} between the interfaces is plotted against the mean value VO_2 L·min^{-1}. Each dot in this graph corresponds to a participant of the study. The dots presented on the lower area of the graph (1.0 – 2.0 L.min^{-1}), represent moderate intensity, while the dots presented at the higher area (>2.0 L.min^{-1}), represents severe intensity.

4.3.2 Effects of varying SIT training protocols _Results

There was a significant increase in endurance capacity test end time in group 2 post training (2.1±1.7 min, p <0.05) while no significant change was seen in group 1 (-0.09±-0.55 min) or the control group (0.1±-1.58 min) (see Table 10). Such significant changes in performance were consecutive to only 9 minutes of exercise over a two-week period. Furthermore, a significant change in VT1 was found between the training groups (group 1: 4.90±0.61 mL·kg^{-1}min^{-1}, p<0.05; group 2: 2.38±1.94 mL·kg^{-1}min^{-1}, p<0.05) and control group (-1.69±0.73) post training. There were however no significant changes between the two training groups.

Data is plotted in Table 10 below; there were no significant changes found in VO_{2peak}, peak watts, VT2, VT2 watts, VT1 watts, Heart rate max and V_E between the training groups and

between the training groups and control. Although not statistically significant, there were increases in VO_{2peak}, VT1 Watts, VT2, and VO_2 $L \cdot min^{-1}$/ $mL \cdot kg^{-1} min^{-1}$and Wingate Fatigue Index Watts in the training groups while there was either a decrease or little change in the control group. As shown in figure 15, a significant difference (p=0.006) was found in the ECT, between the low frequency/high volume group versus the high frequency/low volume group and control. Additionally, there was a significant difference (p=0.002) in VT1 (expressed in $mL \cdot kg^{-1} min^{-1}$) between the training groups and control.

Figure 15 - Performance test results

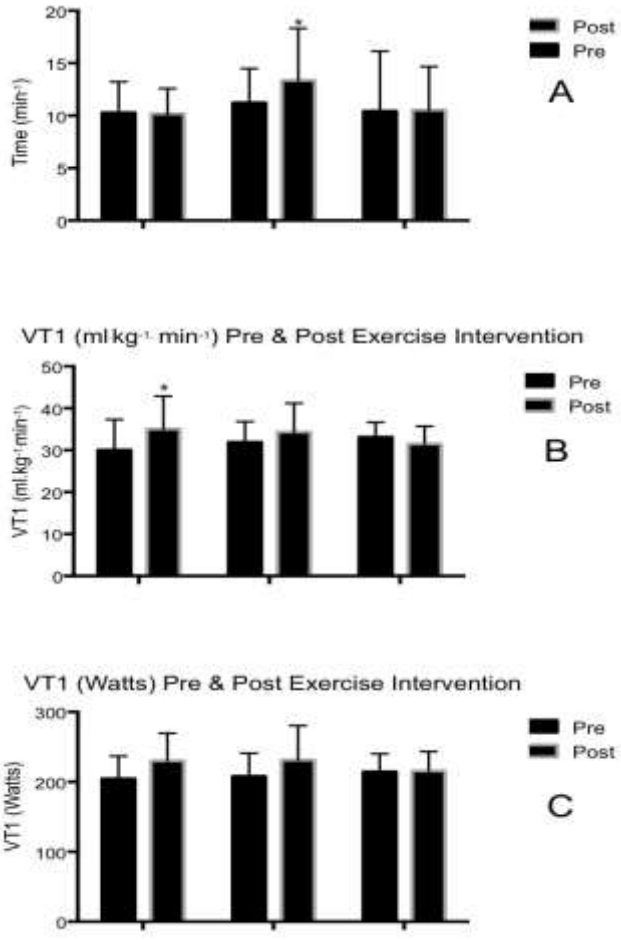


Fig 15_ ECT measures showing comparisons between pre and post exercise interventions (G1: group 1; G2: group 2; C: control): (A) Endurance capacity test, p = 0.006 (B) 1st ventilation threshold VT1 $mL \cdot kg.min^{-1}$, p = 0.002. (C) VT1 Power output at Watts pre- and post-exercise intervention, p = 0.275.

Table 10 - Physiological and performance measures

Variable	Group 1			Group 2			Control			P Value
	Pre	Post	Δ	Pre	Post	Δ	Pre	Post	Δ	
VO_{2Peak} ($L \cdot min^{-1}$)	3.80 ± 0.46	4.02 ± 0.48	**0.22↑**	3.92 ± 0.48	3.93 ± 0.46	**0.01↑**	3.93 ± 0.45	3.84 ± 0.47	**0.09↓**	**0.144**
VO_2Peak ($mL \cdot kg^{-1} \cdot min^{-1}$)	48.11 ± 7.42	50.64 ± 6.92	**2.53↑**	50.37 ± 5.97	51.20 ± 5.89	**0.84↑**	50.09 ± 5.29	49.06 ± 4.87	**1.03↓**	**0.094**
***VT 1 ($mL \cdot kg^{-1} \cdot min^{-1}$)**	**30.07 ± 7.30**	**34.97 ± 7.91**	**4.90↑**	**31.96 ± 4.93**	**34.33 ± 6.87**	**2.38↑**	**33.14 ± 3.55**	**31.46 ± 4.28**	**1.69↓**	**0.010**
Watts at VT1	204.44 ± 32.45	230.00 ± 39.69	**25.56↑**	207.78 ± 33.45	231.11 ± 49.36	**23.33↑**	214.29 ± 25.73	215.71 ± 27.60	**1.43↑**	**0.060**
VT 2($mL \cdot kg^{-1} \cdot min^{-1}$)	42.70 ± 7.06	44.94 ± 7.11	**2.24↑**	44.42 ± 5.61	45.66 ± 5.85	**1.23↑**	43.06 ± 4.12	41.96 ± 6.06	**1.10↓**	**0.176**
Watts at VT2	307.78 ± 34.56	325.56 ± 28.77	**17.78↑**	314.44 ± 41.57	328.89 ± 37.56	**14.44↑**	305.71 ± 31.55	320.00 ± 40.00	**14.29↑**	**0.080**
HR max ($b \cdot min^{-1}$)	180.00 ± 10.00	183.00 ± 12.00	**3.00↑**	183.00 ± 12.00	184.00 ± 13.00	**1↑**	180.00 ± 10.00	181.00 ± 11.00	**1.00↑**	**0.072**
Peak Power (Watts)	394.44 ± 35.39	402.22 ± 38.66	**7.78↑**	391.11 ± 43.43	403.33 ± 40.93	**12.22↑**	400.00 ± 40.00	422.86 ± 40.71	**22.86↑**	**0.340**
VE_{Peak} ($L \cdot min^{-1}$)	160.26 ± 25.62	157.96 ± 19.96	**2.30↓**	148.53 ± 17.11	154.87 ± 23.51	**6.33↑**	145.20 ± 33.77	145.53 ± 34.69	**0.33↑**	**0.522**
Wingate Fatigue index (W/s)	24.44 ± 3.25	31.22 ± 5.57	**6.8↑**	26.25 ± 4.48	27.79 ± 4.29	**1.5↑**	28.94 ± 3.92	28.28 ± 3.63	**↓0.66**	**0.092**
***ECT (min)**	**10.27 ± 2.96**	**10.18 ± 2.41**	**0.09↓**	**11.42 ± 3.27**	**13.52 ± 4.97**	***2.10↑**	**10.43 ± 6.1**	**10.53 ± 4.1**	**0.1↑**	**0.008**

↑=Increases in value, ↓=decreases in value, VT1 = Ventilation threshold one, VT2 = Ventilation threshold two. Results are reported in Averages (AVE) and Standard (SD) Deviations. *Variables that showed significant changes (p value < 0.05).

4.4 Discussion

4.4.1 Optimal ECT apparatus sub-study _Discussion

This sub-study found that there were no significant differences between the mouthpiece and the facemask over a ventilation range of 10 – 120 $L \cdot min^{-1}$. Also, the values for endurance time, ventilation, VO_2 and VCO_2 were not significantly different when using the mouthpiece versus the facemask. These values contrast with findings from Saey et al. (2006) who found the mouthpiece yielded higher ventilation and gas exchange parameter values than the facemask. A possible explanation for these conflicting results may lie in the difference in subject demographics between our study and previous literature. All subjects of the present study were healthy young males with ventilation rates that reached or exceeded 120 $L \cdot min^{-1}$, whereas Saey et al. (2006) utilised a chronic obstructive pulmonary disease (COPD) patient population with maximum ventilation rates of 50 $L \cdot min^{-1}$.

Furthermore, the results of this study showed a strong level of agreement in the Bland-Altman plot during high intensity testing and a low level of agreement during low intensity testing. However, the latter result remained within the 95% limit of agreement. This result again differed from the findings of Saey et al. (2006) who demonstrated a poor level of agreement during low intensity testing. A respiratory burden due to an added inhalation resistance by the facemask (Krustrup, 2004) may have contributed to both studies showing lower levels of agreement in the Bland-Altman plot during low intensity testing. This added resistance combined with the well-documented increase in expiratory resistance in COPD patients (Nicholls et al., 2008) may explain why the Bland-Altman plot findings from Saey et al. (2006) found that the facemask and mouthpiece with nose clip could not be used interchangeably during low intensity testing for gas exchange. Conversely, the present study's high level of agreement with the Bland-Altman plot at high intensity testing could be explained by the increased respiratory rate and greater recruitment of accessory respiratory muscles which occur during high intensity exercise compared to low intensity exercise (Brooks et al., 2004). Furthermore, a study by Dooly et al. (1996) showed that lactate thresholds and ventilation thresholds appears to be the same when using a facemask as opposed to a mouthpiece. Based on these results, it is likely that the facemask and

mouthpiece with nose clip can be used interchangeably when executing performance testing on healthy subjects reaching higher ventilation rates.

Previous research has suggested that by using a mouthpiece and nose clip, the low level of comfort might cause a premature end to the test (Bloch et al., 1995). This data did not coincide with the findings of the present study, as there was no significant difference in end-exercise time between the mouthpiece and facemask. Nor was there any systematic difference in the endurance time, as the difference in endurance time between the mouthpiece and the facemask was within the range of the test-retest variability of the endurance time when two sub maximal cycling exercises are performed in the same condition (Van't Hul et al., 2003). This indicates that the choice of interface had an insignificant impact on the determination of endurance time. In conclusion, this sub-study supports the use of the facemask as a valuable alternative to the mouthpiece in the determination of the endurance time to constant-work rate exercise in healthy subjects aiming to achieve high intensities. Caution must however be used when fitting the facemask as type, size and placement of the mask may have a significant impact on the results. All the subjects of the following research henceforth used the more comfortable facemask during testing.

4.4.2 Effects of varying SIT training protocols _Discussion

The purpose of this study was to determine if a sprint interval training (SIT) intervention, consisting of 18 sprint intervals (a total of nine minutes of training) delivered over two weeks at two different regimes could improve cycle endurance capacity (ECT) and assess which delivery method was most efficacious. High frequency/low volume (group 1) gained significant increases in VT1, and although not significant, increases in VO_{2peak} and VT2 which have been shown in the literature to indicate gains in oxidative capacity and endurance performance (Laursen & Jenkins, 2002). Contradictory to this, the high frequency/low volume group showed no gains in ECT. This is a surprising result as the vast majority of energy utilised during the ECT is supplied from oxidative processes (Gibala et al., 2006). Moreover, the low frequency/high volume group (group 2) also had significant increases in VT1, and although not significant, increases in VO_{2peak}, and VT2. However, the low frequency/high volume group also had a significant increase in ECT. Whilst both groups

showed increases in oxidative capacity parameters, these results indicate that performing nine repetitions of SIT one time per week for two weeks is more efficacious than three repetitions of SIT three times per week for two weeks, when aiming to increase cycle endurance capacity.

One can only speculate as to the potential mechanism responsible for the significant improvement in ECT in the low frequency/high volume group (group 2) and the greater change in particular to VT1 in the high frequency/low volume group (group 1). Burgomaster and colleagues (2006), in their study found that the same type of training (30s sprints with 4 minutes recovery) as performed by the high frequency/low volume group in this experiment (although 1-3 reps more per session) stimulated an increased fractional muscle oxygen extraction and decreased net muscle glycogenolysis and lactate accumulation through biochemical adaptations. Moreover, increased activity in the enzyme citrate synthase repeatedly appears as a contributing factor to adaptation in muscle O_2 extraction (Barnett et al., 2004; Burgomaster et al., 2005; Burgomaster et al., 2006; Bailey et al., 2009). Furthermore, another study which has implemented a larger number of repetitions (8 – 12, 30 second sprints), similar to this experiments group 2, found that VT1, VT2 and aerobic capacity also increased significantly (Laursen et al., 2005). Therefore, it is a unique finding that while the other studies mentioned have found physiological and metabolic changes with both high and low frequency reps, this study is the first to show a difference in performance outcomes comparing the same training volume and different regime in trained individuals.

Interestingly, the low frequency/high volume group (group 2) had a significant increase in time to fatigue, while the high frequency/low volume group (group 1) showed no change. The Wingate's Fatigue Index provides a possible explanation of this finding, where the high frequency/low volume group fatigued quicker (6.7 W/s) than the low frequency/high volume group (1.5 W/s). Though this finding is not statistically significant, it can be hypothesised that the high frequency/low volume group suffered from increased phosphocreatine depletion and lacked the adaptations necessary for H^+ removal. It has been argued that the substantial increase in muscle and blood H^+ accumulation following sprint training may in turn impair sprint-repeatability performance (Bishop et al., 2011). In fact, an

explanation to the high frequency/low volume group's increase in anaerobic threshold parameters during a maximal oxygen consumption test and a lack of change during endurance capacity test, may be that they simply had an increase in neuromuscular efficiency only and no change in metabolic efficiency. Neuromuscular efficiency improves by muscle fibre recruitment (Enoka & Fuglevand, 1993; Enoka, 1997; Aagaard et al., 2002). During continuous tension on the muscle, signals are being transmitted via the efferent and afferent nerves (Enoka & Fuglevand, 1993; Enoka, 1997). When the intensity is greater the nerves recruit more fibres to help with the stimulus (Enoka & Fuglevand, 1993; Enoka, 1997; Aagaard et al., 2002). Greater strength is achieved when more fibres are recruited (Enoka & Fuglevand, 1993).

This is consistent with other findings stating that if athletes are unable to activate a muscle maximally by voluntary command as needed during SIT sessions, an improvement in this capacity will increase strength (Enoka & Fuglevand, 1993; Enoka, 1997) and in return affect the cyclists power output as well at the anaerobic threshold. Moreover, it is well known that when training reaches an intensity of >95% of maximum power, neuromuscular components are being affected (Bishop et al., 2011; Girard et al., 2011; Buchheit & Laursen, 2013). Additionally, it has been argued that changes in pulmonary VO_2 measurements could be the consequence of the increased recruitment of fast twitch fibres in fatigued situations (Saunders, 2004). In contrast to the high frequency/low volume group (group 1), the low frequency/high volume group (group 2) did nine repetitions as opposed to three; that is, nine sprint bouts and nine recovery bouts. During the recovery bout of an interval session the heart rate declines at a proportionally greater rate than the return of blood to the heart, resulting in a brief increase in stroke volume (the amount of blood the heart pumps with each beat) (Bogdanis et al., 2007). This increase places an overload on the heart, which strengthens it and enables the skeletal muscles to be cleared of waste metabolites quicker owing to the elevated rate of blood flow when there is little demand for activity from the tissues (Bogdanis et al., 2007). The presence of high stroke volume peaks during the recovery interval and because there are many recovery intervals during the low frequency/high volume group's interval workout, stroke volume peaks many times providing a stimulus for improving maximum stroke volume and thus improving the capacity of the oxygen transport system (Bogdanis et al., 2007). Also during the recovery intervals a

significant portion of the muscular stores of quick energy—adenosine triphosphate (ATP) and phosphocreatine (PC)—that were depleted during the VO_2 work period are replenished via the aerobic system to again be available as an energy source (Bogdanis et al., 2007). Furthermore, metabolic efficiency can be defined as the ratio between the total amount of effective mechanical work done by the muscles and the energy expended by the body (Gaesser & Brooks, 1975; Moseley et al., 2004). Metabolic efficiency in cyclists has been stated to range from 18 – 23%, where an improvement in cycling efficiency indicates an increase in mechanical power output for any specific metabolic cost (Gaesser & Brooks, 1975; Coyle et al., 1992). Essentially, this suggests that with 18-32% of metabolic energy expenditure, a change in efficiency leads to 28% more power than an individual that has lower efficiency (Moseley et al., 2004). Moreover, during a one hour cycling performance test, only a 1.8% difference in gross efficiency can result in as much as a 10% difference in maximal sustained power (Horowitz et al., 1994; Moseley et al., 2004). Therefore, the conclusion can be drawn that the subjects from the low frequency/high volume group gained a central adaptation and became more metabolically efficient after their training intervention.

However, the subject demographic must be taken into consideration, as they all were trained individuals. If the training intervention were performed on an untrained population, most likely there would be more significant changes in the assessed parameters. Furthermore, with a higher number of subjects participating in the study, the widest difference in results between groups might have become significant. Therefore, further investigations should be performed on a larger number of participants in order to confirm these current findings. Also, further investigation should be carried out at the genetic level, where one might find that genes related to parameters such as oxidative capacity and enzyme activity might have been expressed in group 1. Additionally, one might find that these genes have returned to baseline due to lack of further stimuli.

The results of this study have an inordinate practical validity and should be of great interest to coaches when designing a training program. The training regime of the high frequency/low volume group might be an efficient training method earlier in the annual plan when the aim is to increase the athletes' VT1, power and neural efficiency.

Furthermore, at a later stage during the annual plan the training method of low frequency/high volume group might be a time efficient method to specifically prepare for endurance events through increasing the athletes' time to fatigue.

4.5 Conclusions

This research innovatively suggests that as few as nine repetitions of 30 s intense sprints once a week for two weeks does increase physiological performance markers in trained cyclists. Furthermore, the results of this study suggests that doing these nine sprints once a week, rather than divided into three times a week, will not achieve the highest performance marker results, but could provide a possibly greater advantage in an endurance event through an elevated tolerance to high intensity exercise over a longer period of time. Further investigations into the mechanism of these results are valid and investigation of parameters such as gene expression differences between the two groups would clarify the results further.

Chapter 5 - Global Gene Expression Changes caused by Sprint Interval Training Intervention

5.1 Introduction

Gene expression in human white blood cells has been utilised as a mirror to assess the response to stressors such as training (Büttner et al., 2007). Furthermore, it has been proposed that the level of expression of certain genes is workload dependent (Büttner et al., 2007). Therefore, the future of assessing gene expression signalling mechanisms in response to training has a huge potential in the area of individualized training program design. Already there is evidence that training stressors induce inflammatory reactions of the immune system, where the activation of the pro and anti-inflammatory pathways are dependent upon exercise intensity and duration (Ostrowski et al., 1999; Büttner et al., 2007). With this information alone, the potential to use the immune system as a physiological indicator of a person's ability to recover from stressors induced by various workloads seems very probable (Büttner et al., 2007; Nieman et al., 2007). Moreover, the field of predicting gains in performance using molecular classifications is progressing rapidly and reported more and more in the literature (Tremblay et al., 1994; Bouchard et al., 2000; Bouchard & Rankinen, 2001; Liew et al., 2006; Timmons et al., 2010). However, limited research exists on white blood cells and gene expression in response to exercise in trained individuals. Thus, the major limitations when performing investigations on trained individuals are to determine the appropriate sample-size and the anticipated effect size.

When planning a study that involves microarray analysis, the investigators must be aware of the challenges involved in estimating the necessary statistical power to find a real biological effect. In terms of statistical power and expected error rates, the sample size is a critical determinant (Page et al., 2006). In order to calculate the required sample size, there needs to be an estimate of the variability in the independent variable. Usually this information is determined by a pilot study or from similar data found in the literature (Page et al., 2006). Through these methods, empirically driven and theoretically defensible sample sizes can be estimated (Page et al., 2006). In studies using gene expression, estimating sufficient sample

size to achieve adequate statistical power is critical when designing this kind of study (Hong, 2012). Furthermore, statistical power is the probability of rejecting the null hypothesis (H_0), while showing that the alternative hypothesis is true (Hong, 2012). However, aspects such as sample size and effect size affect the null hypothesis. Unless the estimation of statistical power is performed at the planning stage, time and resources might be wasted. Furthermore, a sufficient sample size can be defined as the minimum number of samples that achieves adequate statistical power for a given effect size (Hong, 2012). It is important to ensure that studies are not under or over-powered, which would result in wasted time and money.

The aim of this study was to investigate SIT and its effect on physiological and biochemical adaptation and regulation in relation to athletic performance. Focus was to be on those genes differentially expressed after SIT together with additional genes that could have a biological basis in the regulation and adaptation that result in changes in performance. The aim was also to investigate the changes in expression levels in response to varying levels of exercise (dependent on frequency and number of sessions) and determine which of these exercise deliveries would achieve a higher predicted performance outcome on the molecular level. Furthermore, the intention was to evaluate if these molecular responses would produce markers related to the phenotypic results in order to suggest that the white blood cell is a potential tissue for reflecting and monitoring changes in performance. By answering these questions, an increased understanding of the physiological and biochemical molecular adaptation of a Wingate test protocol and SIT would be obtained. Furthermore, it would provide sport coaches, exercise physiologists, sport scientists and athletes another perspective on the use of gene expression profiling in training prescription and SIT for the improvement of aerobic performance.

5.2 Methods

Trained cyclists aged 18-35 years old (n=26) were randomly assigned to either group 1 (n=9) or group 2 (n=9) and carried out performance tests before and after the 2-wk sprint interval training intervention. A control group (n=8) carried out the performance tests 2-wks apart from each other without doing any training intervention. All subjects had a minimum of three sessions on the cycle ergometer per week. After a routine medical screening, all the subjects were informed about the procedures and protocols used in the study. This project was approved by BUHREC at Bond University; ethics number RO1063.

Testing: A maximal oxygen consumption test (VO_2max), an endurance capacity test (ECT) and a Wingate test were performed 3 days prior and 3 days post training intervention.

Training intervention: Each training session consisted of repeated 30 s all out efforts on an electronically braked cycle ergometer (Lode, Groningen, The Netherlands). During the 4 min recovery between each 30 s test, the subjects remained on the ergometer and continued cycling at a low cadence. G1 group attended the laboratory for training three times per week on Monday's, Wednesday's and Friday's for two weeks. G2 group trained once per week on Wednesday's for two weeks.

Blood collection: Approximately 8mL of venous blood was sampled from an ante-cubital vein into an EDTA tube on three separate time-points over the two weeks. Blood samples from subjects were collected into 4mL EDTA blood tubes. Peripheral blood mononuclear cell extraction was carried out over Ficoll-Paque and the protocol consisted of the following: Within a 14 mL Falcon tube, 4 mL Gibco PBS (pH 7.4 sterile) was added to 4 mL of fresh blood. Tubes were then inverted 3 times and 8 mL of diluted blood was layered over 4 mL of Ficoll-Paque. Post centrifugation at 900 RCF for 20 min/room temperature, plasma was removed gently (stored at -80°C) and WBCs were collected using with a 1000µL pipette (filter tipped) and spun down at 4°C/4000 RCF/10 min, rinsed in PBS (pH 7.4) and finally stored at -80°C for further processing. Blood was collected and analysed at baseline, 2

weeks post baseline (immediately post the last session in week 2) and 72 hours post training week 2.

Cell Isolation: In order to prevent alteration of phenotype or function of the mononuclear cell population during density gradient centrifugation, Ficoll-Paque PLUS was used. Anti-coagulated blood (4mL) was diluted with PBS. Ficoll-Paque PLUS (1.3mL) was allocated into six culture tubes with 2.3mL of diluted blood layered over it and the culture tubes were centrifuged three times. Finally, the white blood cells were then collected and placed into Eppendorf tubes and centrifuged. Ficoll-Paque PLUS permits the separation of mononuclear cells from other elements found in the blood during centrifugation due to its specific make up.

Total RNA Purification: The TRizol® Plus RNA purification kit (Invitrogen Australia Pty Limited) was used to isolate total RNA. Briefly, WBC pelleted sample tubes were removed from an -80°C freezer and placed at room temperature with TRizol® (Invitrogen). Passaging the sample through a pipette tip several times performed lysis of cells. Samples were then incubated for 5 min at room temperature to allow for the complete dissociation of nucleoprotein complexes. 0.2 mL chloroform was then added and gently mixed by hand for 15 s. Following a 3 min incubation (room temperature), samples were centrifuged at 12,000 x g for 15 min at 4°C. A colourless upper phase containing the RNA was collected gently and transferred into a new tube containing an equal volume of 70% ethanol. Samples were vortexed for 30 s and placed above RNA Spin cartridges. Binding of RNA, washing steps and elution series were undertaken following manufacturer's instructions (TRizol® Plus RNA purification kit, Invitrogen Australia Pty Limited). The RNA was eluted into 100 µL of pure water and quality control was established on RNA samples. Assessment of both RNA concentration and purity was undertaken with the NanoDrop 1000 Spectrophotometer. RNA quality was assessed using the measurement of 260/280 ratios (Appendix G).

Genome-Wide Microarray: Microarrays were performed using Illumina whole-genome gene expression beadchips consisting of oligonucleotides immobilized to beads held in microwells on the surface of an array substrate. Data quality and reproducibility are supported in part by the high-level bead-type redundancy on every array. The present study used the new

HumanHT-12 v4.0 Expression BeadChip that supports highly efficient human whole-genome gene expression profiling studies. Microarray experiments profiled n=6 of group 1 and n=4 of group 2 participants both at baseline (before training intervention) and immediately post training intervention (Figure 9). Another series of microarray experiments were run to profile n=6 of group 1 and n=3 of group 2 participants at three days post training intervention (Figure 9). Therefore, a total of 29 Illumina based microarrays were performed.

Undertaking PBMC gene expression analysis involves a relatively non-invasive tissue collection that may be used as a surrogate means of obtaining information on transcriptomic regulations following diverse types of stimuli. However, while non-invasive and practical, such an approach introduces potential disadvantages due to the nature of the cells studied. These are numerous types of these blood cells with each performing different functional roles in the blood. As an example, the contribution of neutrophils to innate immunity sees their numbers increase rapidly during infection. It is therefore reasonable to account for their concentration when undertaking a gene expression experiment using PBMCs.

While it is intuitively more accurate to determine gene expression from sub-populations of PBMCs such as T cells or B cells, one should consider the disadvantages of such an approach. Fluorescent activated cell sorting is a standard technique of choice to specifically isolate one type of cell from others. However, this procedure might introduce diverse stimuli during the protocol and subsequently may alter these cells, triggering possible changes in cellular gene expression at the transcriptomic level.

Another less expensive approach is to undertake the PBMC transcriptome expression analysis and determine the cell count of each subpopulation of cells. This cellular count measure could then be taken into account when the gene expression analysis is undertaken.

Statistical analysis of Microarray: The Bioconductor software suite for bioinformatics (http://www.bioconductor.org/) was used to analyse the data with methods sourced from the Limma and Lumi packages. These packages utilize the R software package

(http://www.r-project.org/), which is a language and environment for statistical computing and graphics. Determination of fold gene expression differences within each group across each time-point versus baseline was analysed (Tables 13 and 14). In addition, the significance of differentially expressed genes was adjusted for multiple hypothesis testing using the Benjamini-Hochberg method (Benjamin & Hochberg, 1995). Comparison of the genes whose expression data was found and shared between both groups is tabulated in Table 12.

Quantitative Reverse Transcription Polymerase Chain Reaction: qRT-PCR was performed to determine the gene expression of candidate genes and to compare these expressions with the findings of differentially expressed genes following the microarray based expression study. Post data normalisation and characterisation of these genes with a known temporal gene expression measure across all time-points in the microarrays experiments (2037 genes) required an undertaking of post validation by qRT-PCR across all time-points using three randomly selected genes. Briefly, qRT-PCR required the conversion of mRNA into cDNA by reverse transcription. The cDNA molecules served as templates and were subject to annealing of specific primers previously designed to flank the DNA sequence of the candidate genes under investigation. A PCR experiment was carried out to amplify these genes in the presence of Sybr green, a double stranded DNA binding dye. This PCR experiment is taking place in a real-time PCR device holding a laser that excites the fluorescent dye. Subsequent direct fluorescence emission can be captured and observed on a computer screen in real-time.

The conditions and steps of the qRT-PCR were briefly as follows:

cDNA synthesis

The following procedure for synthesis of cDNA was undertaken. Briefly, in a 200 uL tube the following were added; Random primers (1 uL), dNTPs mix: (1uL), RNA (11uL of pooled RNA - Pool of all control RNA at each time-point or Pool of all sample RNA at each time-point). Samples were briefly mixed and left at 65°C for 5 minutes. 7uL of the following bulk was added; 5XFS buffer (4 uL), 0.1M dTT (1uL), “Rnase out” (1 uL) and Superscript III (1 uL) (SuperScript® III First-Strand Synthesis System kit: Cat nb 18080-051). The preparation was

mixed gently and left to incubate at 25°C for 5 minutes and a reverse transcriptase reaction was undertaken at 50°C for 1 hour. The reaction was then terminated at 70°C for 15 minutes and the preparation stored at -20° C.

Design of PCR primers specific to particular genes

Using Ensembl DNA sequences (website: http://www.ensembl.org/), PCR primer pairs were designed for several genes including the internal control 18s gene, the interleukin-6 receptor gene, the IL16 gene and the CD69 gene using the bioinformatical tool Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primer sequences are tabulated in Table 11.

PCR amplification: All these primers were used in PCR amplifications together with the diverse existing cDNA (template starting material diluted 25x into DNase/RNase free pure water) from group 1 and group 2 cohorts. The gene 18s was used as the internal housekeeping gene for qRT-PCR data analysis purposes (see Table 11). PCR runs were undertaken in triplicate for each gene using a reaction cocktail (final volume of 25uL) that would include the addition of 12.5 uL of Biorad IQ™Sybr Green® Supermix (cat nb 170-8882), 1 uL of 5 mM of each forward and reverse primer solution, 2 uL of cDNA and finally overlay with pure water to a 25uL final reaction volume. PCR runs were carried out in individual Qiagen/Corbett 0.1 mL strip tubes (Qiagen Type 4 strip Tube & Caps, Bulk, 250/pk cat nb: 981103) previously placed on a minus 20°C pre-cooled metal tube carrier in the following order: cDNA, followed by the resulting PCR mix. Tubes were closed off and manually handled to allow the whole PCR solution to rest at the bottom of the tube. Real time detection of PCR products was performed using the Corbett Research Rotor-Gene 3000 (Qiagen-Corbett, Corbett Rotor-Gene 6000). All PCR tubes were placed in the 72 well Rotor within the real-time PCR machine. The following cycling conditions were inputted in the Rotor Gene software: Cycle 1, 94^{o}C 12 min (x1), Cycle 2, 94^{o}C 30 s, 59^{o}C 30 s, 72^{o}C 30 s (x45). A post-PCR amplification protocol was pre-set before the run to obtain melt curve representations (ramping from 50° to 99°C with 1°C ramping every 5 seconds).

Data Analysis: Following qRT-PCR analysis, CT values from triplicates were collected at linearity and used to calculate the 18S rRNA corrected CT (or ΔCT) for each gene. Triplicate repeats of ΔCT were assigned with ± SE determinations and were then used to calculate the

mean corrected difference in CT between controls and samples (or ΔΔCT ± SE). The extent of the response is determined by 2 mean (ΔΔCT), while a negative value suggests repression of gene expression, so the relative degree of response is calculated by $2^{-\text{mean }(\Delta\Delta CT)}$.

Table 11 – Primer design for microarray validation of genes ILR, IL16, and CD69

Gene	Forward Primer	Reverse Primer	# Bases
18s	5'CTTAGAGGGACAAGTGGCG3'	5'GGACGTCTAAAGGGCATCACA3'	F_19 R_21
IL6R	5'CACGACTCTGGAAACTATTCATGCTA3'	5'GGGAACATCCACCAGCAAGT3'	F_26 R_20
IL16	5'TCTGCAGCCAGTGATGTTTC3'	5'GAGGCTTGTCTCCGTGTAGG3'	F_20 R_20
CD69	5'TGCCATCAGACAGCCATGTT3'	5'TGACCACTTCCATGGGTGAC3'	F_20 R_20

Power analysis: The online power calculator; *Sample Size Calculation for Completely Randomized Treatment-Control Designs by Mei-Ling Ting Lee* (Figure 16) was used to determine which level of sample size this study should have based on the current findings. A completely randomized design was used for this power study due to the study's equal number of treatment and control conditions without using matched pairs (Lee & Whitmore, 2002). This calculation computes the required sample size n for completely randomized designs in which differential expression between n treatment units and n control units is of interest. Furthermore, the total number of experimental units for this calculation is 2n (Lee & Whitmore, 2002). The following is a demonstration on how the power calculator can be used to anticipate study n=number, with a sliding scale to assess how n=number changes according to alpha level and mean difference in log-expression (Lee & Whitmore, 2002).

Input parameters were as follows:

E(R0): Mean number of false positives. The number of false positives is equal to alpha (α), that is the confidence level or probability of rejecting the null hypothesis. This alpha ranges from 0-1, where 0.05 has been the most used probability in studies related to this area. By entering a value of 1-5 into the power calculator, there will be a limit of 1-5 false positives out of 100 genes (Lee & Whitmore, 2002).

G_0: Anticipated number of genes in the experiment that are not differentially expressed. Due to the thousands of genes that may be involved in a microarray study, the anticipated number of genes that are NOT differentially expressed is unknown. Therefore, this is a number that must be anticipated prior to the conduction of the study. Based on the five other studies investigating gene expression changes in the white blood cells through microarray, the average number of differentially expressed genes is 301 genes at the acute time-point (Laursen et al., 2002; Laursen & Jenkins, 2002; Connolly et al., 2004; Laursen et al., 2005; Radom-Aizik et al., 2008). This study found a total of 2037 genes and in order to anticipate the number of un-differentially expressed genes to add into the power calculator, the number 301 was subtracted from 2037 (2037-301= 1736) (Lee & Whitmore, 2002).

Power: The specified power level for an individual gene, which represents the expected proportion of differentially expressed genes that will be declared as such by the tests, has not been mentioned in the previous literature. However, this study initially used a power of 80% (Lee & Whitmore, 2002).

μ1: Mean difference in log-expression between treatment and control conditions as postulated under the alternative hypothesis H1. Based on the current literature an arbitrary fold change (FC) cut-offs of >2 seems to be used (Lee & Whitmore, 2002). Due to the limitations of the subject population and the tissue adaptations that are of interest, the expected fold changes were small and have therefore in this study been chosen to be 1.5 (Lee & Whitmore, 2002).

σd: Anticipated standard deviation of the difference in log-expression between treatment and control conditions. The standard deviation of the gene expression fold changes found from this study cannot be used owing to the very small changes in subjects and groups. Additionally, this value has not been reported in any of the current and relevant papers. Accordingly, this current anticipated standard deviation of the difference in log-expression value has been adapted from the study of Lee & Whitmore (2002) and is 0.3 (Lee & Whitmore, 2002).

Figure 16 - Public calculator available for sample size calculation

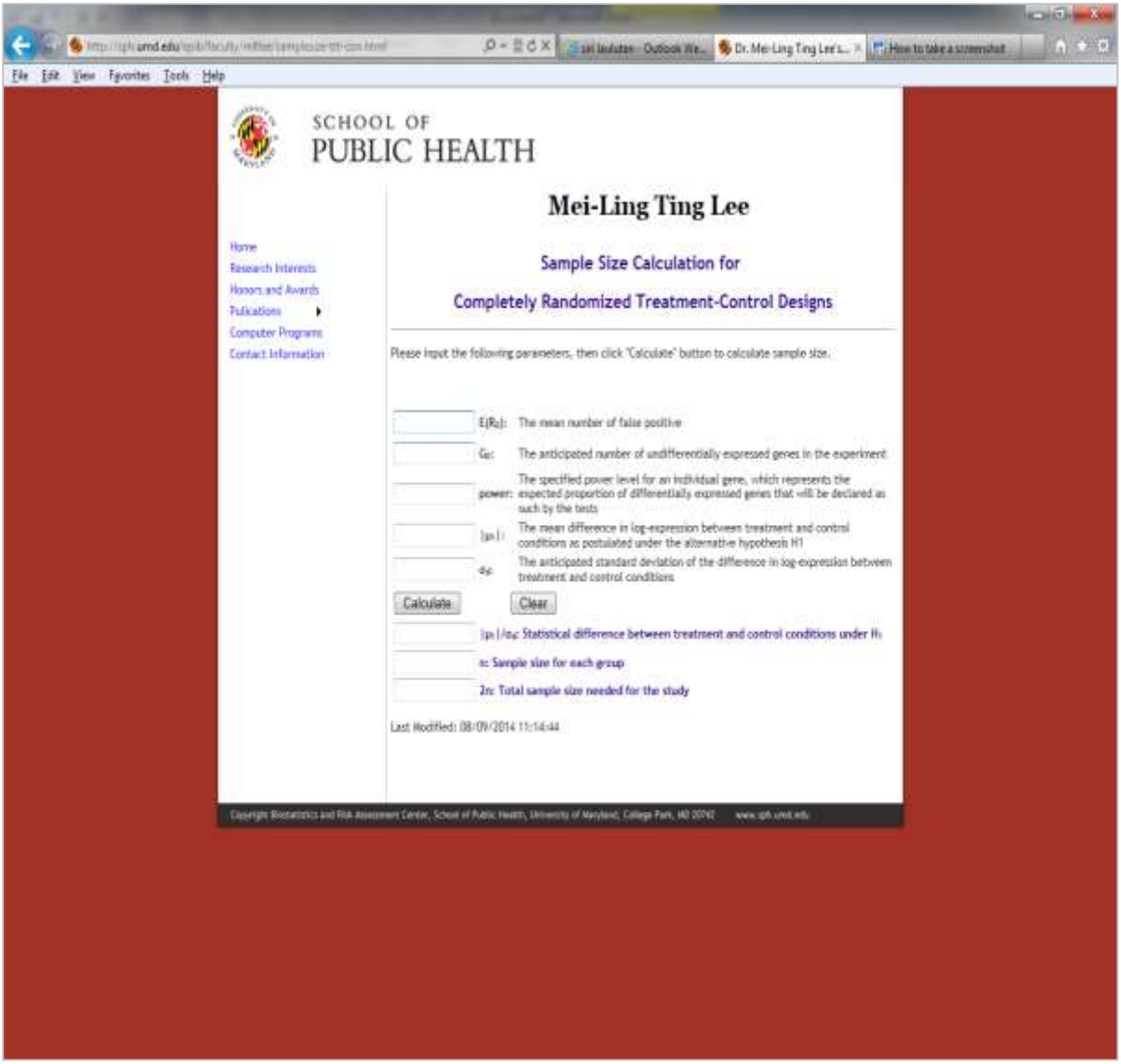


Fig 16 _ http://sph.umd.edu/epib/faculty/mltlee/samplesize-trt-con.html

5.3 Results

The results of this study show no significant changes in white blood cell gene expression at any of the time-points within each group (time-points 1-2, 2-3 & 1-3) ($p > 0.05$). Furthermore, the results showed no significant changes in white blood cell gene expression in between groups (high frequency/low volume - group 1 and low frequency/ high volume - group 2) within any of the time-points (see Table 12). 2037 genes were registered; however, the average p-value corrected for multiple hypotheses testing across time-points and between groups was 0.97 (see Tables 12, 13, 14). In fact, the lowest p-value found across the time-points and groups was $p = 0.11$ (see Table 14).

Table 12 - Top 20 genes, comparison of G1 vs. G2 at each time-point

	Baseline		Acute time-point		Delayed time-point	
	Group 1(n=6) vs. Group 2 (n=4)		Group 1(n=6) vs. Group 2 (n=4)		Group 1(n=6) vs. Group 2 (n=3)	
Gene	Fold change	P-value	Fold change	P-value	Fold change	P-value
KARS	0.97	1	1.09	1	0.72	1
TCTEX1D2	0.9	1	1.14	1	0.72	1
TAF1B	0.76	0.39	1.07	1	0.81	1
CSTF2	1.11	1	0.82	1	1.45	1
ATP5C1	0.65	0.92	1.02	1	0.58	1
ISCA1L	0.88	1	1.23	1	0.89	1
UQCC	1.16	1	0.97	1	1.47	1
APRT	0.99	1	1.05	1	0.71	1
ING1	0.76	0.59	1.01	1	0.68	1
SLC35F2	1.13	1	1.2	1	0.74	1
PTK2B	1.11	1	0.93	1	1.54	1
SBDSP	0.89	1	1.16	1	0.68	1
42248	0.7	0.48	1.07	1	0.79	1
WDR75	1.19	1	1.09	1	1.46	1
RPL36AL	0.78	1	1.22	1	0.79	1
LOC653471	0.98	1	0.93	1	1.06	1
HAX1	0.97	1	1.19	1	0.81	1

Top 20 genes retrieved from the statistical analysis of this study: gene name, fold changes and adjusted p-values are shown.

Table 13 - Top 20 genes, pairwise comparisons of time-points for group 1

	Group 1		Group 1		Group 1	
	Baseline vs. Acute		Acute vs. Delayed		Baseline vs. Delayed	
Gene	Fold change	P-value	Fold change	P-value	Fold change	P-value
KARS	0.98	1	0.88	1	0.86	1
TCTEX1D2	0.94	1	0.97	1	0.91	1
TAF1B	1.06	1	0.89	1	0.94	1
CSTF2	0.98	1	0.99	1	0.97	1
ATP5C1	1.12	1	0.76	1	0.85	1
ISCA1L	1.11	1	0.8	1	0.89	1
UQCC	0.98	1	1.13	1	1.11	1
APRT	1.16	1	0.85	1	0.99	1
ING1	0.98	1	0.93	1	0.91	1
SLC35F2	0.97	1	0.95	1	0.92	1
PTK2B	1.04	1	0.99	1	1.03	1
SBDSP	1.15	1	0.82	1	0.94	1
42248	0.98	1	0.8	1	0.79	0.95
WDR75	0.87	1	1.21	1	1.05	1
RPL36AL	0.92	1	0.93	1	0.85	1
LOC653471	0.86	1	1.21	0.79	1.03	1
HAX1	0.92	1	1	1	0.92	1

Table 14 - Top 20 genes, pairwise comparisons of time-points for group 2

	Group 2		Group 2		Group 2	
	Baseline vs. Acute		Acute vs. Delayed		Baseline vs. Delayed	
Gene	Fold change	P-value	Fold change	P-value	Fold change	P-value
KARS	1.1	1	0.58	0.11	0.64	0.79
TCTEX1D2	1.19	1	0.62	0.11	0.73	0.92
TAF1B	1.51	0.25	0.67	0.11	1.01	1
CSTF2	0.72	0.77	1.75	0.13	1.26	1
ATP5C1	1.75	0.77	0.43	0.13	0.75	1
ISCA1L	1.55	0.71	0.58	0.13	0.9	1
UQCC	0.82	1	1.72	0.13	1.41	0.96
APRT	1.24	1	0.58	0.13	0.72	1
ING1	1.3	0.89	0.63	0.13	0.82	1
SLC35F2	1.04	1	0.59	0.15	0.61	0.79
PTK2B	0.87	1	1.64	0.15	1.43	0.93
SBDSP	1.5	1	0.47	0.17	0.71	1
42248	1.5	0.73	0.6	0.17	0.9	1
WDR75	0.8	1	1.61	0.18	1.29	1
RPL36AL	1.44	0.77	0.6	0.18	0.87	1
LOC653471	0.81	0.77	1.37	0.18	1.11	1
HAX1	1.13	1	0.68	0.18	0.77	1

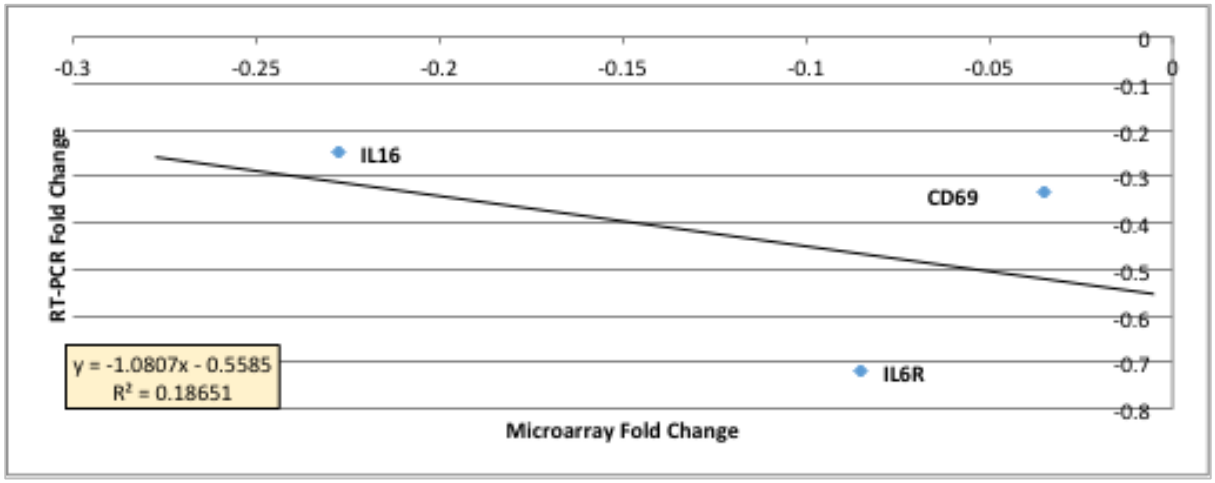
As shown in the Tables 12-14, the p-values indicate that there is no significant difference between time-points which suggests that, if there are differences to be found, they are likely small and a larger sample size is required to detect that level of effect. These findings are confirmed with the results of the qRT-PCR validation study results (see Table 15).

As shown in Table 15, the qRT-PCR fold changes of CD69, IL16 and IL6R, were very low ranging from -1.45 to 0.88 in group 1 and -1.84 to 0.97 in group 2, with no significant p-values. Some of the validation points were consistent between qRT-PCR and microarray; such as in group 1 CD69 baseline versus acute with -0.40 and -0.53, and group 2, baseline versus acute IL16 with -1.22 and -0.08. Additionally, there were consistencies with positive direction changes such as group 2 baselines versus acute IL6R at 0.20 in qRT-PCR versus 0.41 in microarray. There are however some small fluctuations in the results between the two types of analyses where one analysis shows a change in the opposite direction to that revealed in the other analysis. These differences were very small, such as at baseline versus delayed in group 1 where IL16 was 0.03 in RT-PCR versus -0.35 in microarray. Due to these small discrepancies, a regression analysis has been performed between the qRT-PCR results and microarray results. The regression analysis produced a correlation coefficient of 0.43, which is very close to 0.5, a moderate correlation (Table 16). This regression was however calculated from statistically insignificant data. The p-value for the correlation was 0.71 ($p>0.05$), a non-significant result, however this could be influenced by the use of only 3 data points. Therefore, definitive conclusions as to the validity of the microarray results cannot be drawn despite the regression analysis results suggesting a correlation between the RT-PCR and microarray findings. Due to these findings, sample size calculations were made with sliding scales in order to assess the current power of the study and how many subjects are needed in order to find significant results.

Table 15 – qRT-PCR Validation results and comparison with Microarray.

	Group 1				Group 2			
	Baseline vs. Acute		Baseline vs. Delayed		Baseline vs. Acute		Baseline vs. Delayed	
Gene	Fold change	P	Fold change	P	Fold change	P	Fold change	P
CD69								
RT-PCR	-0.40	0.64	-0.82	0.88	0.92	0.68	-1.04	0.97
Microarray	-0.53	0.99	0.28	0.99	-0.17	0.99	0.28	0.99
IL16								
RT-PCR	0.50	0.61	0.03	0.51	-0.30	0.53	-1.22	0.86
Microarray	-0.37	0.99	-0.35	0.99	-0.11	0.99	-0.08	0.99
IL6R								
RT-PCR	0.22	0.56	-1.45	0.97	0.20	0.56	-1.84	0.88
Microarray	-0.31	0.99	-0.35	0.99	0.41	0.99	-0.09	0.99

Table 16 – Regression analysis of qRT-PCR and Microarray data



The sliding scales demonstrate how the sample size varies according to alpha and mean difference in log-expression between treatments. Furthermore, it shows that the initial power calculation undertaken for this present study was appropriate. However, several factors were not considered in this present study such as: individual intrinsic biological variability of subjects and the consideration of an untrained group with past history of some sort of collateral level of 'trained' lifestyle. These unknowns can be the determinant points resting in non-high levels of significance in the array results. According to the sliding scale in Figure 17, an expected mean difference in log-expression of 1.5, with an 80% power per gene would need only 5 subjects per group if the mean number of false positives was set to 1 and 3 equally, while only 4 subjects would be needed if the false positive number was set

at 5. As this study's highest mean difference in log-expression was only 1.1, the sample size needed became an unrealistic 78 – 98 respectively depending on the accepted false positive (see Figure 17). If the power per gene were changed to 90% as shown in Figure 18, this study would need a sample size of 97-119 subject depending of the accepted false positive (see figure 18). This finding should make it very clear to other researchers that overall statistical power of exercise based gene expression studies should be one of their higher priorities in order to produce statistically significant data. Some calculations of power are provided below in Figures 17 and 18.

Figure 17 - Sliding scale of sample size calculations with 80% power

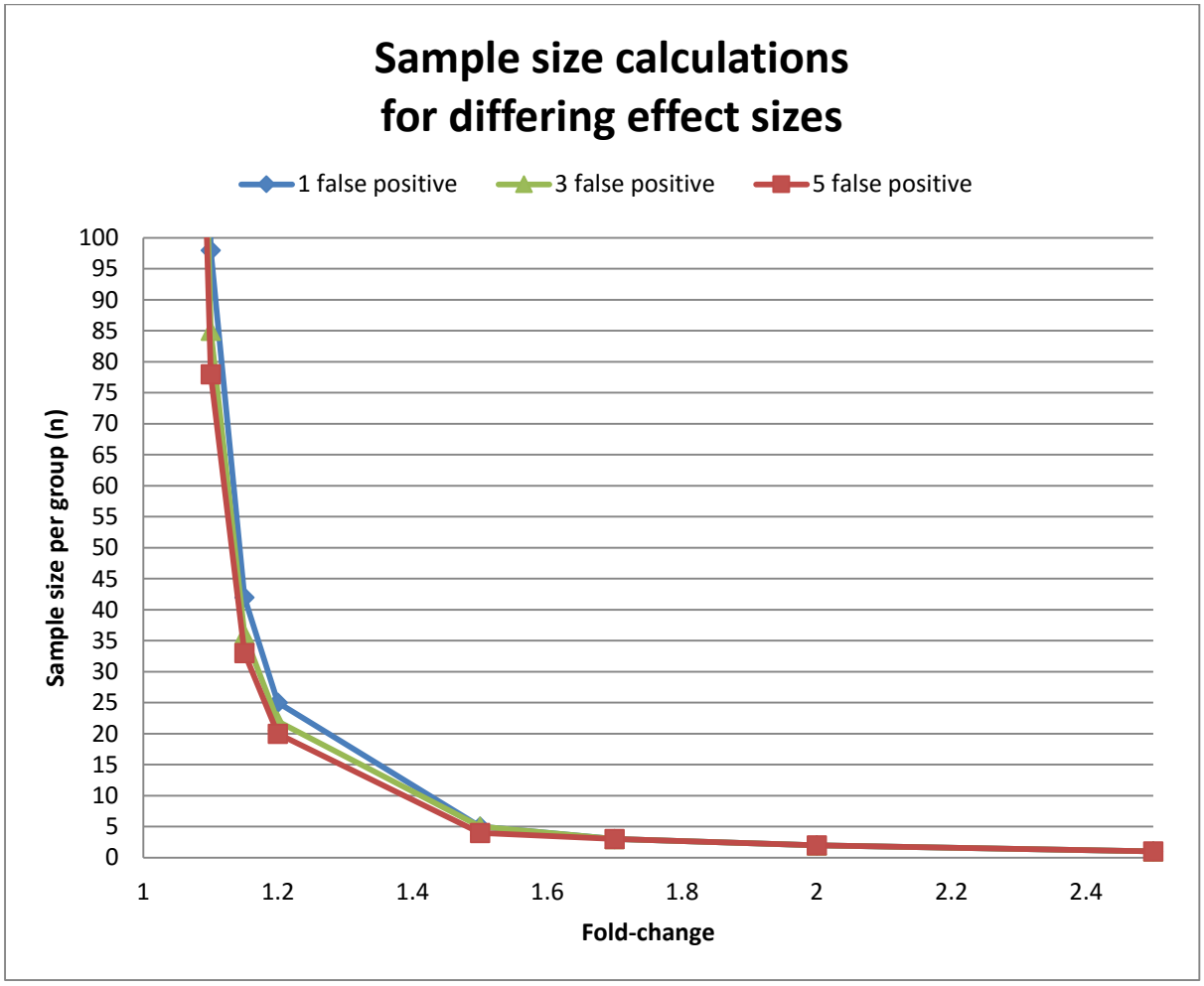


Fig 17_ Factors that remain unchanged on the sliding scale: # total genes NOT differentially expressed (G_0) 1736, Power for each gene 80%, Standard deviation 0.3

Figure 18 - Sliding scale of sample size calculations with 90% power

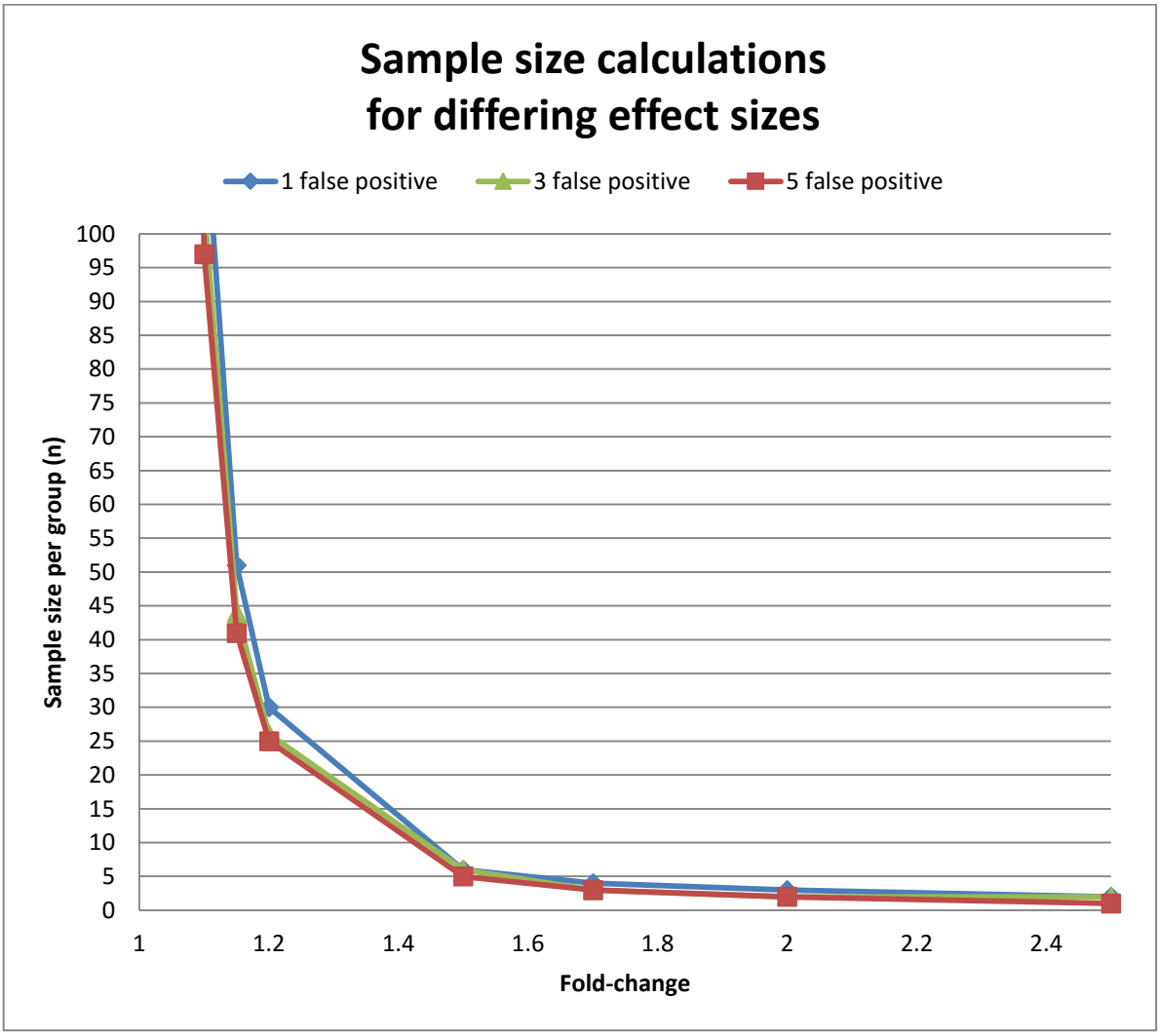


Fig 18 _ Factors that remain unchanged on the sliding scale: # total genes NOT differentially expressed (G0) 1736, Power for each gene 90%, Standard deviation 0.3

5.4 Discussion

The protocol of exercise frequency and intensity in this current study has revealed significant changes in physiological performance with only 9 minutes of exercise. The distribution of this type of training is therefore influencing performance, however no significant results were detected in gene expression within this study. The timing of the sample collection in this project was comparable to previous studies that had reported some statistically significant results. It is therefore probable that the non-significant gene expression profile observed in this present study is not explained by the timing of sample collection. Hence, the reasons for these insignificant results are suggested to be twofold;

firstly, the subject number may have been too low in order to meet the required statistical significance, and secondly, as the study involved a trained population, the individual variations were too large which created a great variance in gene expression within subjects across the groups. However, it must be reiterated that the exercise protocol employed was sufficient to produce improvements in performance, with these changes presumably being due to underlying alterations in gene expression in muscle tissue. However, these changes in gene expression were not evident in leucocytes.

Regarding the potential issues with sample size and power; the sample size of n=9 in the training groups and n=8 in the control group showed significant results in the performance parameters. However, various limitations have occurred which compromised the integrity of the extracted RNA (see limitations of the study p. 168). Consequently, several samples could not be hybridised and therefore were not able to be integrated in our analysis. As a result, the training groups only comprised an n number of n=6 (group 1) and n=4 (group 2). These groups were analysed in the present gene expression study.

The initial power study for the project, regardless of the limitations encountered during this thesis, showed that by using repeated measures ANOVA, with 2 time periods and 3 treatments, a number of 12 subjects were required in each tested group to reach a power of 80%. As a matter of comparison, obtaining blood from only 5 subjects within each group for gene expression analysis will ultimately reach a power of 58%. Additional factors that affect power and sample size conclusions include variability of the population studied, the desired detectable differences, the power to detect differences and an expectable error rate (Dupuy & Simon, 2007; Hong & Park, 2012). Literature that has assessed sample size for detecting gene expression in microarrays has stated that statistical power means the probability that the tests will detect genes that are truly differentially expressed (Wei et al., 2004; Dupuy & Simon, 2007; Hong & Park, 2012). However, if the n=number is low there is an increased number of false positive genes found. The increased number of false negatives due to a low n=number decreases the chance of finding genes that relate to the biological differences that the experiment is looking for (Wei et al., 2004; Dupuy & Simon, 2007). Due to a frequent case of low statistical power combined with large false positives in microarray

analysis studies, the between study reproducibility is very low (Wei et al., 2004; Dupuy & Simon, 2007; Hong & Park, 2012).

The extent, nature, and sources of variation in gene expression among healthy and trained individuals are a fundamental and unexplored aspect of human biology (Whitney et al., 2003). In order to understand potential applications of gene expression in exercise, an understanding of their normal variation within and between individuals over time and with age, gender and fitness level is needed. Peripheral blood is an accessible source of cells with which to investigate these variables. Furthermore, circulating leucocytes have now in the literature been characterised as "scouts", continuously maintaining a vigilant and comprehensive surveillance of the body for signs of infection or threats of any sort (Zeller & Blankenberg, 2013). Additionally, it has been suggested that gene expression in the circulating leucocytes can potentially provide an early warning of the threats they discover (Zeller & Blankenberg, 2013). In contrast to this, studies have now shown evidence of distinct patterns of inter-individual and temporal variation in gene expression as a result of physiological responses associated with differences in the cellular composition of the blood sample with gender, age and time of day the sample was drawn (Dumeaux et al., 2010; Zeller & Blankenberg, 2013). Even the handling required for isolation of the mononuclear cell fraction from whole blood may create changes in gene expression. Actually, a common variable in studies with peripheral blood is the time difference between phlebotomy and sample utilization. Additionally, the variation in length of time elapsing between the sample collection and the RNA purification has been found to create individual differences in gene expression. It is clear that the need to minimize handling time when analysing peripheral blood by microarrays is critical (Whitney et al., 2003; Dumeaux et al., 2010).

As mentioned above, due to the individual variability in gene expression, the importance in having a sound project designed towards discarding such variability is of high priority and important to address for all exercise science research. Although the "n" number of this study was small, the other components and design surrounding this project was tightly designed and carried out. All the subjects were thoroughly screened and familiarized according to the guidelines of the American College of Sports Medicine (Thompson et al., 2009), to ensure they were all healthy and capable of taking part in the study. Blood was

collected from all subjects at the same time-point in the training intervention (pre-training intervention, 10 minutes after last session and after three days of recovery) and the blood was processed within two hour of collection, collected by the same person at all time-points for every subject. After the samples were processed, they went through a very stringent protocol of Illumina whole-genome gene expression beadchips which consist of oligonucleotides immobilized to beads held in microwells on the surface of an array substrate (Schulze & Downward, 2001; Korol, 2003). Furthermore, data quality and reproducibility is supported by the high level bead-type redundancy on every array (Schulze & Downward, 2001; Korol, 2003). A final process in this analysis also validates the hybridization performance of every bead on every BeadChip, ensuring 100% quality control (Schulze & Downward, 2001; Korol, 2003). Additionally, Illumina expression bead chip arrays are arranged in a multi-sample format for higher through put and reduced sample-to-sample variability (Schulze & Downward, 2001; Korol, 2003). This present study used the new HumanHT-12 v4.0 Expression BeadChip that supports highly efficient human whole-genome gene expression profiling studies. Its content provides a genome-wide transcriptional coverage of well-characterized genes, gene candidates and splice variants, with a significant portion targeting well-established sequences supported by peer-reviewed literature. Each array on this BeadChip targets more than 47,000 probes (Schulze & Downward, 2001; Korol, 2003).

Data output from the Limma software package show box-plots displaying the distribution of intensities for each array. These were generated to investigate any differences in spread across the arrays. Normalization was performed to reduce the variability in the data due to non-biological sources and due to these stringent procedures the resulting normalized data-set has a much more consistent distribution of intensities with less variability from non-biological sources. The data finally went through stringent statistical methods using the Lumi package. This includes a variance-stabilizing transformation (VST) algorithm that takes advantage of the technical replicates available on every Illumina microarray. The quality control of a Lumi Batch object includes a data summary (the mean and standard deviation, sample correlation, detectable probe ratio of each sample (microarray)), divergent quality control plots, and the control probe information. The quality control plots include: a density plot, a box plot pairwise scatter plot between microarrays or a pair scatter plot with

smoothing, a pairwise MAplot between microarrays or a MAplot with smoothing and a density plot of coefficient of variance. Finally, p-values describing the significance of differential expression of genes were adjusted for multiple hypothesis testing using the Benjamin-Hochberg method (Benjamin & Hochberg, 1995).

Conclusively, quantification of steady-state mRNA levels by reverse transcription polymerase chain reaction (qRT-PCR) was completed after microarray analysis in order to validate the gene expression analysis and candidate findings of the microarray. The technique is highly sensitive, permitting analysis of gene expression from very small amounts of RNA (Freeman et al., 1999; Bustin & Mueller, 2005). The qRT-PCR analysis performed in this study showed very low fold changes of CD69, IL16 and IL6R, ranging from -1.45 to 0.88 in group 1 and -1.84 to 0.97 in group 2, with no significant p-values. These results are consistent with the fold changes registered for the same genes in the microarray analysis and confirm the non-significant results of the microarray analysis. Only three genes were used for a Sybr Green based real-time PCR validation of gene expression and the data obtained from real-time PCR showed large p-values. To possibly account for better statistical significance in the comparison of the microarray and real-time datasets, TaqMan based technology and more candidate genes (e.g. n=6-10) should have been performed for better validation, interpretation and statistical significance validation.

One might also consider if the training intervention would not be significant enough to cause the hypothesised changes in gene expression. This could be due to the fact that the training intervention had merely a total of 9 minutes exercise time over the 2 weeks. However, that is not the case as this study showed a significant difference $p<0.05$ in anaerobic threshold between the training groups and control group and a significant difference in time to fatigue (ECT) between the high frequency/low volume (G1) vs. the low frequency/high volume (G2) and control group. This shows that this studies training protocol produced changes in endurance performance in already trained individuals. The fact that these adaptations in performance have occurred means that changes must have occurred at the molecular level. As discussed in the literature review, the fact that short programs of SIT modify performance though molecular, biochemistry and physiological factors has been extensively suggested in the literature already (Burgomaster et al., 2005;

Burgomaster et al., 2006; Gibala et al., 2006; Burgomaster et al., 2007; Burgomaster et al., 2008). However, this study is novel in that it shows how frequency of SIT sessions differently affects performance. Furthermore, this study is novel in terms of the low number of total repetitions shown to alter performance in trained individuals. Due to these findings one might wonder the reason for no possible trend between subjects in the recorded gene expression data. A few areas that are interesting to explore in relation to this study's findings is "responder" versus "non-responder" status, as well as attenuation of genes as a response to adaptation. These are two areas that have been recently explored related to training adaptation and seem to be logical topics to include in the discussion of this study's results.

High responders, low responders and attenuation

Factors such as age, nutritional aspects and genetic predisposition has been found to affect the hypertrophic response, although they are highly variable between individuals (Blair et al., 1996; Hittel et al., 2005). For instance, the phosphorylation of the translation initiation protein p70 S6 kinase (S6K), which is involved in protein translation, has been found to correlate with muscle hypertrophy that occurs with resistance training (Tusher et al., 2001; Timmons et al., 2006). This indicates that local influences regulating muscle protein synthesis, as opposed to systemic factors, are of dominant importance in determining hypertrophy (Tusher et al., 2001; Timmons et al., 2006). However, it has been found that training status, exercise adherence, nutritional support and age are subject to large variation in training adaptations as a response to certain type of exercise (Hawley et al., 2006). This might be explained by noncoding RNA which has emerged in recent years (Saxena et al., 2006) as being important for skeletal muscle biology (Gluckman & Hanson, 2007; Nagano et al., 2008).

In particular, microRNAs (miRNAs) are accepted regulators of mammalian cell phenotype (Baldwin & Krebs, 1981; Hamilton & Booth, 2000; Nickenig et al., 2002). miRNAs are 22-nucleotide posttranscriptional regulators of gene product abundance that are able to block the translation of a protein-coding gene (Keller et al., 2007). Furthermore, as miRNAs regulate development and differentiation (Dennis et al., 2003; Mahoney et al., 2005), it is interesting to know that brain and skeletal muscle tissue have the most tissue specific

miRNA species (Jørgensen et al., 2007). miRNAs have also been implicated in the regulation of metabolism (Dennis et al., 2003; Choe et al., 2005), insulin secretion (Larsson et al., 2005), and muscle disorders (Gesta et al., 2006; Chen et al., 2008), including type 2 diabetes (Jørgensen et al., 2007). miRNA are considered to be significant regulators of muscle protein expression and when assessing human miRNA *in vivo* it appears that it may affect protein synthesis, rather than mRNA stability (Gluckman & Hanson, 2007). Several miRNAs are highly regulated *in vivo* and *in vitro* during muscle development and, in turn, regulate muscle differentiation (Bouchard et al., 1995). As not all subjects demonstrate a robust physiological adaptation or molecular response to exercise training, it is important to link acute changes in gene expression with the phenotypic outcome relevant to the intervention (Strohman, 2003; Snyder et al., 2009). In the area of endurance training this has been clearly demonstrated, however it is also reasonable to suspect a similar scenario in resistance training. Therefore, due to a lack of knowledge on individual adaptation to chronic training, the acute molecular response to acute exercise may not be informative. Additionally, it has been well accepted that progressive resistance training creates gains in skeletal muscle mass (Timmons et al., 2005). However, this responsiveness seems to be very variable between individuals. It has been suggested that factor such as sex, age, diet, physical activity level and previous training status affect the degree of muscular adaptation (Timmons et al., 2005; Frazer et al., 2009). Interestingly, even when controlling several of these variables, it has been shown that considerable variation in the training adaption still occurs (Blair et al., 1996; Hittel et al., 2005; Hawley et al., 2006). With this in mind, it is important to suggest that further study is needed in order to define factors, which significantly influence the variation in muscular adaptation between individuals.

Attenuation of gene expression is also an interesting hypothesis to the lack of significant results in this study's data. Transcriptional attenuation is a regulatory mechanism that causes premature termination of transcription under certain conditions, thereby preventing expression of the mRNA required for expression of the corresponding gene products. One might hypothesise that due to the trained population in this study, a negative feedback mechanism would exist in the tissue of some of the subjects to reduce the level of gene expression as exercise adaptation has already occurred. This concept is supported in a study by Richardson et al. (2000) who found a significant increase in vascular endothelial growth

factor (VEGF) mRNA in untrained human skeletal muscle after a single bout of exercise (Richardson et al., 2000). However, the authors found the VEGF mRNA in trained individuals to be attenuated (Richardson et al., 2000).

This finding supports the notion that increased levels of mRNA are attenuated when adaptation has occurred, suggesting a negative feedback mechanism (Richardson et al., 2000). This indication is further supported by Starkie et al. (2001) who suggest that carbohydrate ingestion attenuates the exercise-induced increase in plasma IL-6 (Starkie et al., 2001). Furthermore, they suggest that the plasma IL-6 concentration might be related to the degree of metabolic stress associated with the relative workload of the exercise session (Starkie et al., 2001). However, the reason as to why carbohydrate ingestion blunts the plasma IL-6 has not been answered. This research suggests that further study should be done on attenuation of gene expression in response to training adaptation in trained and untrained individuals, both between groups and within groups.

Other studies

Due to the interesting findings in the literature which conflict with the data of this project, it is of great interest to consider other studies and their methods in order to assess why they found significant changes in active/trained individuals using a low “n” number. As with any other study, this study was based on the current literature available at the time. Furthermore, at this point in the study it is important to return to the literature used as inspiration for the hypothesis and assess if the discoveries made are consistent with their results. In this case, the gene expression results of this project in contrast with most of the findings on the same topic. For this reason, it is important to discuss the differences between these results and the work of others. Studies selected were due to the impact they had on the choice of thesis topic. Additionally, these studies were included due to their low “n” number, assessed tissue, study intervention and outcome.

Current research is very limited in the area of wide genome microarray analysis on human white blood cells and its response to exercise. Only four studies have investigated this area to date. However, only two of the studies seems to have some consistency in their gene

findings, sharing seven genes (Connolly et al., 2004; Radom-Aizik et al., 2008). Both of these studies isolated and analysed the PBMCs, while the two other studies did not mention if they looked at total white blood cells or a specific part of the blood composition. Additionally, Connolly et al. (2004) and Radom-Aizik et al. (2008) have a very similar subject population as well as exercise intervention. Their subject population was healthy males, who conducted a 30-minute exercise bout. It is well known in the literature that individuals unaccustomed to exercise would produce a much higher response molecularly, as well as physiologically, compared to trained individuals (Laursen et al., 2002; Laursen & Jenkins, 2002; Laursen et al., 2005).

Furthermore, the one study (Zieker et al., 2005a) investigating "trained athletes", did so with a very heterogeneous subject population. Firstly, 33 years separated the youngest and oldest participant. Their average training volume per week was separated by 85 km, while their half marathon running time varied from 77 minutes to 139 minutes. It is well known in the literature that the aging process is associated with a significant decline in skeletal muscle and thus encompasses the effects of altered central and peripheral nervous system innervations, altered hormonal status and inflammatory effects (Doherty, 2003). This by itself makes this study's subject population questionable. Additionally, as there seems to be a large variability in training status between these subject populations, their response to a half marathon will be very different. The individual running an average of 110 kilometres per week would most likely not find a half marathon to be a challenge. This assertion is based on the fact that this person has the lowest running time in this event. Furthermore, the subject with the lowest average training time per week (also the oldest) might have done a personal best when running the half marathon in 129 minutes. Additionally, their physiological and molecular response to this intervention would be very different. Indeed, in previously untrained individuals, exercise induced stress increases blood flow, oxygen delivery, oxygen extraction and fat metabolism in working muscle (Laursen & Jenkins, 2002). These factors change as a person adapts to exercise. However, when training becomes habitual as a response to large training quantities, further exercise induced stress becomes a challenge without changing mode of training (Laursen & Jenkins, 2002). Therefore, in the study of Zieker et al. (2005a), the molecular stress placed on this heterogeneous subject population should in theory be very variable. In contrast to this, the subject population of

this project was matched for physical characteristics, weekly training volume and intensity, with opposing results. Considering the varying and transient results in the literature compared to this thesis, further study is justified on this topic. Furthermore, in order to create a reproducible project, there seems to be a few factors that may be critical to a successful result. These factors consist of 1) a higher "n" number, 2) the use of technical replicates and 3) more sound reporting of project variability.

Limitations of the study

Performing a training study with humans is never an easy task in itself due to the dependence on individual's commitment to the study and their time availability. In any research containing human subjects, this point seems to be a limitation for most. Additionally, when performing a study investigating the trained population, it is challenging to convince participants to commit to the training intervention. This is merely due to an additional level of training placed onto their current program. This added training might not fit into the subjects, or their coaches training plan. However, commitment to a training regime, coach or both is vital in order for an athlete to gain performance changes. Due to this limitation, the optimal n=number is a challenging segment of a training study to obtain.

Additionally, when collecting blood from trained subjects, another limitation presents itself. Due to anecdotal evidence presented to potential subjects in regard to the donation of blood and associated declines in performance during preparation for competitions, the scepticism as to whether they should participate in such a study is great. This finding is based on feedback from potential and participation subjects of this study. It needs to be stated that the mature male human body has a blood volume of 4.7-5 litres. Furthermore, it has been found that endurance trained individuals have approximately 35-40% higher total haemoglobin and blood volume than untrained individuals (Heinicke et al., 2001). Therefore, extracting 8 - 16 mL of blood will not affect their performance. In fact, plasma will be replaced within 24 hours. Due to these challenges, the "n" number of this study became a major limitation. Working with trained individuals, phonotypical performance changes are already small. For these reasons, the initial calculation of power for the study is important. Although the initial power calculation of this study showed that by analysing blood from 5 individuals, there would be a 58% chance the samples would show differentially expressed

genes. It is a possibility that the power calculation used initially did not suit for analysis of blood in a trained population. The sample size and power should be calculated by using previous experiments or a pilot study in order to estimate the experimental error standard deviation (σ) of gene log-expression (Lee, 2014). The calculation of the standard deviation of the difference in log-expression between intervention and control conditions is then given by $\sigma_d = \sqrt{2}\,\sigma$ (Lee, 2014).

It has presented itself in the literature that time of day of phlebotomy may affect the gene expression in the white blood cell due to circadian rhythm (Dumeaux et al., 2010; Zeller & Blankenberg, 2013). This is another limitation of this study, due to the work and training commitment of the participating subjects. Although the time course between testing, training days, training session and blood collection was kept consistent within and between subjects and groups, the time of day when testing, training and blood collections were performed could not be controlled.

White blood cells (WBC), or leucocytes, are nucleated blood cells that carry out the immune functions of the body. Leucocytes are the most transcriptionally active cells in blood and are the focus of most blood gene expression studies (Zheng et al., 2006). Leucocytes are made up of granulocytes, lymphocytes and monocytes (Zheng et al., 2006). Granulocytes can be further divided into neutrophils, basophils and eosinophils (Zheng et al., 2006). Lymphocytes can be divided into B-cells, T-cells and natural killer (NK) cells (Zheng et al., 2006). The number of cells in each leucocyte subtype can change significantly during states, such as inflammation (Zheng et al., 2006). The inter- and intra-subject variation is an important issue to consider for blood gene expression studies (Whitney et al., 2003; Fan & Hedge, 2005). Inter-subject variations may be due to differences in demographics such as age, gender, ethnic background, health and nutritional status, metabolism and medical history (Whitney et al., 2003; Fan & Hedge, 2005).

A study of inter-subject variations in PAXgene-stabilized whole blood using Affymetrix gene chips found that the most variable genes among 32 healthy subjects are predominantly immunoglobulin variable region or related genes (Fan & Hedge, 2005). Intra-subject variations come from biological influences within the body such as hormone variation and

diurnal changes (Whitney et al., 2003). Intra-subject variation of ribosomal and protein synthesis genes in human blood gene expression has been reported (Whitney et al., 2003). A recent study of mouse transcriptome profiles suggests that about 10% of the genes in various mouse tissues have a circadian rhythm in their mRNA expression (Rudic et al., 2005). Accurate analysis of *in vivo* gene expression in blood cells may be complicated by changes in gene expression after phlebotomy caused by sample collections, handling, storage and uncontrolled coagulation (Rainen et al., 2002). Intracellular RNA might be rapidly degraded ex vivo by specific or non-specific endogenous nucleases (Rainen et al., 2002). Furthermore, unintentional gene expression might be induced as a sensitive response of blood cells to environmental changes such as temperature (Rainen et al., 2002).

Buffy coat preparations contain most of the leucocytes in blood with small amounts of contaminating red blood cells and platelets, whereas peripheral blood mononuclear cells (PBMC) contain only mononucleated cells in blood (lymphocytes and monocytes), with very few other cell types (Tamul et al., 1995; Rainen et al., 2002). Therefore, gene expression patterns from fractionated blood will be different from whole blood. This is not only because of differences in blood cell composition, but also because fractionation can reportedly alter the expression of select genes within blood cells (Tamul et al., 1995). RNA processing steps such as reverse transcription, labelling and amplification can introduce further variability (Tamul et al., 1995; Ståhlberg et al., 2004). The reverse transcription is especially problematic as the type and sequence of primers and the choice of reverse transcriptase enzyme and the vendor can influence the final gene expression data (Ståhlberg et al., 2004). This has been an unresolved issue for technologies such as microarray and RT-PCR that rely on reverse transcription.

Messenger RNA extraction protocol

Due to the challenge in getting trained individuals to participate in this study, from when white blood cells were isolated and frozen to when samples were thawed for mRNA extraction could take some time. Samples needed to be prepared in bulk as they were processed externally for genome-wide microarray. How much this time period damaged the mRNA in the sample is hard to say. Furthermore, literature is lacking in this area.

Additionally, as there are four key steps involved in the solid phase extraction procedure; cell lysis, nucleic acid absorption, washing and elution (Gjerde et al., 2009; Nicosia et al., 2010), there are four steps as to where the tissue could potentially be compromised and mRNA degraded. Although all measures possible were made to prevent degraded cells, it is a sensitive protocol to work with. The technology of RNA purification combines the selective binding properties of a silica-based membrane with the speed of microspin technology (Gjerde et al., 2009; Nicosia et al., 2010). A particular high-salt buffer system allows up to 100µg of RNA longer than 200 bases to bind the RNeasy silica membrane (Gjerde et al., 2009; Nicosia et al., 2010). The initial step in this process is to condition the column for sample absorption. This can be done by using a buffer that has a particular pH in order to convert the surface on the solid into a particular chemical form (Gjerde et al., 2009; Nicosia et al., 2010). Originally, this study collected blood from six time-points, however due to financial constraints, three time-points were chosen. Unfortunately, several of the samples did not pass quality control and in the end the project was left with only n=6 in group 1 and n=4 in group 2. Although the search to find the compromising step in the analysis was carried out, the answer has still not been found. However, the laboratory in which the isolation of mRNA was performed had issues with the air-conditioning system. Mold and dust were apparent and some extractions were affected when the extractions were performed nearby this logistic issue. During the extraction, no particular suspicion was made about that probable RNA degradation by nucleases. Recently, this issue was put forward and the air conditioning system was fixed.

5.5 Conclusion

This study confirms the challenges that researchers in this area are faced with. Firstly, research design and planning must become a much more acknowledged focus in the scientific community. Secondly, as the microarray analysis technique can provide genome-wide patterns across different conditions, the significance of these observed differences is important (Pan et al., 2002). It has been found that basing the data on one single array may not be reliable as it might contain a high amount of “noise”. This can be resolved with technical replicates. Technical replicates are two aliquots of the same extraction (Pan et al., 2002). This will ensure precision and allow for testing differences within treatment groups.

In fact, current literature is lacking in this area as few studies have used technical replicates, most likely due to the cost of microarray experiments. Thirdly, research papers have often failed to generate reproducible projects due to the misleading descriptive statistics. If normally distributed, the study results can be described entirely by two parameters, namely the mean and the standard deviation. Standard deviation (SD) is used to describe the variability between individuals in a sample, which means that the higher the SD, the larger the subject variability. Authors however, often overlook this and instead standard error of the mean (SEM) is used. SEM is used to estimate how the mean of the sample is related to the mean of the underlying population which makes the variability within the sample seem much smaller than it really is. This is therefore an inappropriate and misleading method to use in descriptive statistics, which makes a research paper difficult to re-produce. It is clear that project design is an important factor in order to achieve a statistically significant and reproducible study. Bioinformatics and biostatistics should be more valued, particularly due to the expected tsunami of research in the area of genetics.

Chapter 6 – Discussion and Future Directions

6.1 Improved athletic performance in trained cyclists after SIT

There was a significant increase in endurance capacity test end time in group 2 post training (2.1±1.7 min, $p < 0.05$) while no significant change was seen in group 1 (-0.09±-0.05 min) or the control group (0.1±-1.58 min). Furthermore, a significant difference in VT1 was found between the training groups (group 1: 4.90±0.61 $mL \cdot kg^{-1} min^{-1}$, $p<0.05$; group 2: 2.38±1.94 $mL \cdot kg^{-1} min^{-1}$, $p<0.05$) and control group (-1.69±0.73 $mL \cdot kg^{-1} min^{-1}$) post training. There were however no significant changes between the two training groups. There were no significant changes found in VO_2peak, peak watts, VT2, VT2 watts, VT1 watts, heart rate max and V_E between the training groups and between the training groups and control. Although not statistically significant, there were increases in VO_2peak, VT1 Watts, VT2, VO_2 $L \cdot min^{-1}/mL \cdot kg^{-1} min^{-1}$ and Wingate Fatigue Index (expressed in Watts/s) in the training groups while there was either a decrease or little change in the control group. Intense exercise as performed by group 1 in this experiment would stimulate an increased fractional muscle O_2 extraction and a decreased net muscle glycogenolysis and lactate accumulation through biochemical adaptations (Barnett et al., 2004; Burgomaster et al., 2005; Burgomaster et al., 2006; Bailey et al., 2009).

Moreover, increased activity in the TCA cycle repeatedly appears as a contributing factor to the change in muscle O_2 extraction (Barnett et al., 2004; Burgomaster et al., 2005; Burgomaster et al., 2006; Bailey et al., 2009). A larger number of repetitions like those performed by group 2 in this experiment, found that, similarly to performing a fewer number of reps, VT1, VT2 and aerobic capacity increased significantly (Laursen et al., 2005). An explanation of group one's increase in anaerobic threshold parameters during a maximal oxygen consumption test and a lack of change during endurance capacity test may be that subjects simply had an increase in neuromuscular efficiency only and no change in metabolic efficiency. Meanwhile, group 2 gained central adaptations as well as becoming more metabolically efficient.

However, the subject demographic must be taken into consideration that for this project was trained individuals. The limitations of this study are two-fold. Firstly, the subject population for this study was trained individuals. Because of this, the performance changes found will naturally be smaller due to the amount of adaptation already present. Secondly, the n=number is a limitation of this study as a trained population has a larger individual variation as a response to the use of a variety of training programs within the group, as well as the presence of responders and non-responders to the training intervention that was applied.

This research innovatively suggests that as little as nine repetitions of 30 s intense sprints once a week for two weeks do increase physiological performance markers in trained cyclists. Furthermore, the results of this study suggests that by doing these nine sprints once a week, rather than divided into three times a week, will not achieve the highest performance marker results. However, they possibly provide a greater advantage in an endurance event through an elevated tolerance to high intensity exercise over a longer period of time. Further investigations into the mechanisms underlying these results on a larger subject population should be performed. Furthermore, parameters such as gene expression differences between the two groups should be implemented in order to clarify the results further.

6.2 Global Gene Expression Changes caused by SIT

The results of this study show no significant changes in white blood cell gene expression at any of the time-points within each group (time-points 1-2, 2-3 & 1-3) ($p < 0.05$). Furthermore, the results showed no significant changes in white blood cell gene expression between groups (group 1 and group 2) at any of the time-points. 2037 genes were registered, however the average p-value across time-points and between groups was $p = 0.97$. In fact, the lowest p-value found across the time-points and groups was $p = 0.11$.

There are several limitations controlling this study which include; Subject population, prejudice of the athletes, time of day when blood was collected, blood composition and individual variation. However, these limitations can be resolved by further study using a

higher power and investigating gene expression in both muscle and blood (possibly in isolated cell types) at several time-points following an exercise session to first determine how rapidly the molecular response to training diminishes in both tissue types. This may lead to the identification of optimal time-points, which could be used in future research and athlete based testing. Additionally, this study should be performed in healthy, untrained people and in trained athletes separately to see how strong the response is between groups.

6.3 Overall discussion

With very short periods of work, such as with 30 s sprints, a very intense load may be placed on the muscles and oxygen transport organs without affecting the anaerobic process that leads to any significant rise in blood lactate concentrations. Furthermore, when performing interval exercise, the intensity of each bout remains higher than if the work would be continuous. However, in the case of several repeated bouts, the stress becomes very high at the metabolic level. With each successive repetition, the role of aerobic energy increases, particularly if PC has not been fully resynthesized. With this form of interval training, PC is depleted extensively and takes longer to fully recharge. This will also increase lactate values. It is this phenomenon that differentiates the two training groups in this study and may partly explain the phenotypic results. According to the literature, a response to a low number of bouts but high frequency of sessions, training similar to group 1, should have increased their ability to repeat intense accelerations, which means their ability to re-synthesise PC would have increased, along with increased muscle glycogen content, reduced glycogenolysis and lactate accumulation during intense efforts. In contrast, group 1 showed a statistically significant increase in AT, an increase in power at AT and some increases in VO_2max (although not statistically significant), but in return showed no change at all in ECT, which may suggest that other factors must be taken into consideration.

A possibility exists that two weeks of high frequency/low volume SIT was not sufficient to stimulate the aforementioned changes. Instead, changes in performance may be attributed mainly to neuromuscular changes. Thus an improved result in gas exchange may actually be due to changes in neuromuscular efficiency. In contrast, group 2 may have increased O_2 transport efficiency, muscle O_2 kinetics, and mitochondrial phosphorylation speed and

enzyme activity. This is based on the fact that with each successive trial, the role of aerobic metabolism increases together with a decline in performance quality. Indeed, in repeated maximal sprint work, if performance declines the contribution of anaerobic energy is relatively low and the aerobic energy contribution is quite high. Of course, this can only be hypothesised based on the current literature. However, in order to confirm these suggestions, further investigations on this topic should take place. There are studies that have assessed these changes at the molecular level on several types of training regimes in the muscle tissue already. However, no studies have yet investigated SIT and its effect on human white blood cells and whether this tissue can reflect a training response to these protocols. In fact, very few studies have done a thorough investigation of this tissue in relation to wide genome gene expression and exercise.

However, this project did investigate whether a microarray analysis of leucocytes would reflect any of the exercise responses achieved by the two training protocols. However, there were no significant changes at any of the time-points between the two groups, within groups or within each subject. Obviously, as there are significant phenotypic changes from the training intervention, molecular adaptations have occurred. However, due to a low n=number, in a subject population containing natural individual differences, it is challenging to find significant molecular adaptations. Additionally, attenuation of gene expression may have occurred, meaning that although transcriptional changes have transpired, mRNA expression has been stopped due to a sufficient level of the required protein and consequently have not been registered as significantly changed at the chosen time-points. It is clear that a significant amount of research is needed in this area due to these limitations.

Chapter 7 – Overall Conclusions

Traditionally, high volume training loads have been used as the main strategy for training endurance athletes. However, this method often leads to overtraining and/or the development of a performance plateau. Furthermore, when monitoring the performance changes in endurance-trained athletes, measures such as VO_2max, blood lactate and percentages of maximum heart rate have traditionally been used. However, a caveat with current monitoring techniques is that functional adaptations have already occurred at the time of the performance measure. These adaptational changes may be positive, where performance has improved, or the adaptations may be negative and the athlete has already reached a plateau or is overreaching. In order to overcome these challenges, other training and monitoring tools can be implemented. SIT is a training tool that can be used as a valuable method to overcome a training plateau for athletes not achieving the desired gains. Furthermore, monitoring gene expression changes in the white blood cells, which are an easily accessible, non-invasive tissue, can be considered as an alternative approach for detecting possible performance changes in their early stages. By using microarray analysis, discrimination can be made of exercise intensity-dependant gene expression profiles in human leucocytes. Furthermore, mastering and applying this type of technology can achieve a better understanding of the pathways that are associated with a normal state compared to an exercise response. This can provide a tool to diagnose and potentially treat overtraining, exercise-induced immune suppression and to evaluate and make recommendations for the improvement of individual training regimes.

The first part of this study found that as little as 18 repetitions of 30 second sprints, which amounts to 9 minutes of exercise, does in fact produce changes in endurance performance in already trained athletes. Furthermore, this type of training is regime dependant. This research innovatively suggests that as little as nine repetitions of 30 s intense sprints once a week for two weeks do increase physiological performance markers in trained cyclists. Furthermore, the results of this study suggests that doing these nine sprints once a week, rather than divided into three times a week, will not achieve the highest performance marker results, but could possibly provide a greater advantage in an endurance event

through eliciting an elevated tolerance to high intensity exercise over a longer period of time. These findings are of great value to athletes and coaches when preparing for endurance events.

Part two of this study found no significant changes in gene expression within the white blood cells as a response to the applied training. Although this is of no value to athletes, it does add knowledge to the scientific community. These results confirm the challenges that lie within research using trained individuals and may serve as a reminder of the importance of meeting the required subject power in any study.

Future studies should be done with a larger “n” number in order to find a clearer difference between the training regimes. Due to the preliminary nature of the present study, selected time-points were chosen to allow a broad investigation of gene expression changes induced by different exercise regimes. Future studies should therefore look at gene expression in muscle and WBC, assessing isolated cell types at several time-points following an exercise session to determine how rapidly the molecular response to training diminishes in both tissue types. This may lead to the identification of optimal time-points, which could be used for future research and athlete based testing. Furthermore, this should be performed in healthy, untrained people and in trained athletes separately to see how strong the response is in both groups.

Overall, this study has found a tool that can be applied to the fundamental aim of an endurance athlete, which is to improve their ability to physically perform at a high intensity for a prolonged pre-determined period of time. By applying as little as 9 minutes of SIT per week for two weeks, an already well-trained cyclist can further improve performance significantly. Although this study did not succeed in finding these performance changes to be reflected in white blood cells, the results are of great importance to further investigators intending to use this tissue as a monitoring tool.

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Appendix A

HIT training protocols and outcomes from the literature

Title	Author	Year	Subjects (control)	Protocol	Outcomes
Effects of sprint cycle training on human skeletal muscle.	Allemeier et al.	1985	11 males (6 males)	3x30s all out sprints. 20min rest. 2-3/wk x 6 weeks	• No fiber transformations from type I to type IIa. • MHC analysis revealed possible conversions within the fast fiber population.
Extremely short duration high intensity interval training substantially improves insulin action in young healthy males.	Babraj et al.	2009	16 males (9 males)	6 sessions; 4-6 x 30s all out sprints w/4min rec. 2 weeks	A strategy to reduce metabolic risk factors in young and middle aged sedentary populations.
High-intensity intermittent cycle ergometer exercise: Effect of recovery duration and resistive force selection on performance.	Baker et al.	2007	8 males	8x6s sprints w/30s or 60s rec	Power output rely on resistive forces selection and recovery duration, and that high-intensity exercise may provide an alternative to aerobic activity in the management of hypertension
Influence of repeated sprint training on pulmonary O_2 uptake and muscle deoxygenation kinetics in humans.	Bailey et al.	2009	10 males & 6 females (5 males & 3 females	4-7 30s all out sprints 4 min rec x 6 sessions. Endurance trained – same amount of total work	Six sessions of sprint but not endurance • Changes in kinetics consistent with enhanced fractional muscle O_2 extraction • faster VO_2 kinetics • ↑tolerance to high-intensity exercise.
Muscle metabolism during sprint exercise in man: Influence of sprint training.	Barnett et al.	2004	8 males (8 males)	3-6 x 30s all out sprint 3min rec. 3/w for 8 wk	• ↑ muscle oxidative capacity • not glycolytic capacity
How anaerobic is the Wingate test for humans.	Beneke et al.	2002	11 males	1x30s all out sprint	• 31.1% anaerobic alactic • 50.3% anaerobic lactic • 18.6% aerobic
Effects of high-intensity interval training on the accumulated oxygen deficit of endurance –trained runners.	Bickham & Le Rossingol	2004	7 males	30 rep x 4sets x 5-15s (90-100%) sprints w/5min rec. 6/wk	The current AOD method was unable to detect the potential small changes in AOD facilitating the increased time to exhaustion reported for the high intensity test.

Effect of short-term sprint interval training on human skeletal muscle carbohydrate metabolism during exercise and time-trial performance.	Burgomaster et al.	2006	8 males (8 males)	6x30s all out w/4min rec. 2 weeks	• ↑ cycling time trial performance • A closer matching of glycogenolytic flux and pyruvate oxidation during sub maximal exercise.
Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans.	Burgomaster et al.	2005	8 males (8 males)	4-7 30s all out sprints, 4min rec. 3xper week, for 2 weeks.	• Increased muscle oxidative potential • Doubled endurance capacity during intense aerobic cycling
Divergent response of metabolite transport proteins in human skeletal muscle after sprint interval training and detraining.	Burgomaster et al.	2007	8 males (8 males)	4-6 x 30s all out cycling w/4min rec. 3 d/w for 6 weeks	• ↑ in skeletal muscle oxidative capacity • Divergent effects on proteins associated with glucose, lactate and fatty acid transport
Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans.	Burgomaster et al.	2008	20 males and 20 females	4-6 x 30s all out sprints w/4min rec. 3days/wk x 6 weeks. 40-60min 65%. 5days/wk	• A time efficient strategy to -↑skeletal muscle oxidative capacity - Induce specific metabolic adaptations during exercise that are comparable to traditional endurance training
Anaerobic energy provision does not limit Wingate exercise performance in endurance-trained cyclists.	Calbet et al.	2003	10 males	1x30s Wingate Normoxia or hypoxia (10.4 O_2 in N_2 equiv. to altitude of 5300m	Performance during 30s Wingate test in severe acute hypoxia is maintained or barely reduced owing to the enhancement of the anaerobic energy release. The effect of severe acute hypoxia on supramaximal exercise performance depends on training background
Neural, Metabolic, and Performance Adaptations to Four Weeksof High Intensity Sprint-Interval Training in Trained Cyclist.	Creer et al.	2004	8 males (8 males)	4x30s sprints, 4min rec. Biweekly for 4 weeks	• ↑ motor unit activation • ↑ exercise plasma lactate levels • ↑total work output With a relatively low volume of sprint exercise compared to endurance training alone.
AMPK signalling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation.	Chen et al.	2000	11 males & females	1 x 30s sprint	Support the concept that inhibition of ACC is an important component in stimulating fatty acid oxidation in response to exercise and that there is coordinated regulation of nNOSμ to protect the muscle from ischemia/metabolic stress.
Changes in oxidative stress markers and NF-kB activation induced by sprint exercise.	Cuevas et al.	2005	8 males	Day 1; 1x30s print, day 2; 4x30s sprints w/4min rec	HIT induces oxidative stress, as evidenced by non cumulative damage to macromolecules and chances in the glutathione status. The data also shows a rise to an activation of the

					transcription factor NF-kB accompanied by a degradation of IkB
AMP deaminase deficiency is associated with lower sprint cycling performance in healthy subjects.	Fischer et al.	2007	139 males & females	2x30s all out sprints w/20min rec	The approximate 10% lower mean power in healthy AMPD-deficient subjects during a 30s Wingate cycling tests reveals a functional role for the AMPD-1 enzyme in sprint exercise
Short-term high-intensity interval training improves phosphocreatine recovery kinetics following moderate-intensity exercise in humans.	Forbes et al.	2008	3 females & 4 males (1 female & 6 males)	4-6 30s all out 4min rec. 3/wk 2 weeks	Short-term high-intensity training is effective means of increasing functional oxidative capacity in skeletal muscle.
The effect of an aerobic interval training program on intermittent anaerobic performance.	Gaiga & Docherty	1995	13 males (11 males)	3min-to-rest-ratio 3days/wk x 9wk	The type of interval training program used in this study increased aerobic power and also enhanced performance in repeated high intensity, short duration work.
Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance.	Gibala et al.	2006	16 males	4-6 X 30s all out sprints w/4min rec. 90-120min at 65% ET	Time-efficient strategy to induce • Rapid adaptation in skeletal muscle • Exercise performance that are comparable to ET
Brief intense interval exercise activates AMPK and 38 MAPK signalling and increases the expression of PGC-1alpha in human skeletal muscle.	Gibala et al.	2009	6 males	4x30s sprints w/4min rec	• Metabolic remodelling induced • by low-volume intense interval exercise • mitochondrial biogenesis • ↑capacity for glucose and fatty acid oxidation.
Markers of coagulation, fribrinolysis and angiogenesis after strenuous short term exercise (Wingate-test) in male subjects of varying fitness levels.	Gunga et al.	2002	15 males	1x30s Wingate. VO_2max test	It was found that prothrombotic markers and, even more pronounced those of the fibrinolytic system were increased. The study provides evidence that due to intensive short term exercise the balance of haemostatic system is shifted to a higher equilibrium.
Muscle metabolites and performance during high-intensity, intermittent exercise.	Hargreaves et al.	1998	6 males	3x30s Wingate w/4min rec. + 30min at 30-35% peak w/60min rec + 1x30s Wingate	The decline in exercise performance does not appear to be related to a reduction in muscle glycogen. Instead, it may be caused by reduced CP availability, increased H+ concentration, impairment in SR function, or some other fatigue-inducing agent.
Exercise training increases branched-chain oxoacid dehydrogenase kinase contnt in human skeletal muscle.	Howarth et al.	2007	20 males & females	4-6 x 30s all out sprints w/4.5min rec. 3/days/wk. 6 wks. 40-60min 65%. 5days/wk	• ↑protein content of BCOAD kinase - may be involved in the mechanism for reduced BCOADa after exercise training in skeletal muscle • differences in models used to study the regulation of skeletal muscle BCAA metabolism, since exercise training

					was previously reported to increase BCOADa during exercise and decrease BCOAD kinase content in rats
Sprint training effects on muscle myoglobin, enzymes, fiber types, and blood lactate.	Jacobs et al.	1987	7 males & 4 females (4 males & 2 females)	2-6 15s & 30s all out sprints. 45s – 15min rest. 6 weeks	• Muscle myoglobin concentration is not increased • Such training induces cellular adaptations without accompanying performance changes.
Increase in the proportion of fast-twitch muscle fibres by sprint training in males.	Jansson et al.	1990	15 males	2-6 x 15s&30s all out sprints. 15-20 min rec. 2-3 days/wk, 4-6 wks	A change in fibre activation frequency may induce an increased synthesis of type II fibre myosin (fast myosin). Hormonal influences such as enhanced adrenergic stimulation of the muscle fibres cannot be excluded as a contributing factor.
Changes in serum creatine kinase, lactate dehydrogenase and aldolase activities following supramaximal exercise in athletes.	Karamizrak et al.	1994	33 males (30 males)	3x30s all out sprints w/6-8 rec	Increases in serum LD activities of athletes and in CK activities of controls appear to be more pronounced, and increases in serum CK, LD and ALS activities seem to depend more on the duration of exercise than on its intensity.
Influence of high-intensity interval training on adaptations in well-trained cyclists.	Laursen et al.	2005	10 males (11 males)	12x30s all out sprints. 4.5min rec. 2/wk x 4wks	Peripheral adaptations rather than central adaptations are likely responsible for the improved performances witnessed in well-trained endurance athletes
Cross validation of the 20- versus 30-s Wingate anaerobic test.	Laurent et al.	2007	50 males	20s or 30s Wingate	When compared to 30s Wingate, the 20s sprint may be considered a valid alternative when used with the predictive non-linear regression equation to derive the final power output values.
Interval training program optimization in highly trained endurance cyclists.	Laursen et al.	2002	10 males (11 males)	12x30s all out sprints. 4.5min rec. 2/wk x 4wks	Supra maximal HIT can significantly improve 40 km time trial performance.
Acute heat exposure increases high-intensity performance during sprint cycle exercise.	Lacerda et al.	2007	9 males	1 X30s all out sprint	Exposure to hot environment caused an improvement in power output for a single 30-s sprint. This increase was associated with an elevation in plasma ammonia suggestive of an increase in adenine nucleotide loss.
Ergometric and metabolic adaptation to a 5-s sprint training program.	Linossier et al.	1993	10 students	8 – 13 reps x 2sets of 5s sprint cycling w/55s rec between reps & 15min rec between sets. 7 weeks	An appropriate adoptive reaction following high-intensity intermittent training for the slow twitch fibres witch exhibit a greater oxidative capacity.
Effect of short-term high-intensity interval training vs. continuous training on O2 uptake kinetics, muscle	McKay et al.	2009	12 males	8-12 X 1min all out sprints. 1min rec. 90-120min at 65% max	VO2 kinetics was faster during moderate-intensity exercise after only 2 days of training, regardless of the type of exercise program (HIT/END).

deoxygenation, and exercise performance.					
The effect of a brief sprint interval exercise on growth factors and inflammatory mediators.	Meckel et al.	2009	12 males	4 x 250m run on a treadmill at a constant intensity of 80% of individual max, 3min/rec	Changes in the anabolic-catabolic hormonal balance and in inflammatory mediators can be used as an objective tool to gauge the training intensity of different types of anaerobic exercises an training period.
Does power indicate capacity? 30s Wingate anaerobic test vs. maximal accumulated O2 deficit.	Minahan et al.	2007	7 males & 7 females	1x30s Wingate & 120% of peak O_2 uptake to exhaustion	A higher anaerobic power does not indicate a greater anaerobic capacity. Furthermore, the ability to maintain power output during a 30s cycle sprint is related to anaerobic capacity.
Assessment of peak power and short-term work capacity.	MacIntosh et al.	2003	13 males and females	3x30s all out sprint with flying start or stationary start	Higher peak power with stationary starts
Muscle performance and enzymatic adaptations to sprint interval training.	MacDougall	1998	12 males	4-10 x 30s Wingate w/2.5 – 4min recovery. 3 x per week for 7 weeks	• ↑Total work over 30s • ↑Peak power • ↑VO2 max • ↑Maximal enzyme activity
Sprint training increase human skeletal muscle Na+, K+, ATPase concentration and improves K+ regulation.	McKenna et al.	1993	6 males untrained	4-10 x 30s Wingate with 4min recovery. 3 x week for 7 week	• Peak power • ↑Work output • ↓Fatigability • ↑Plasma [K+] • ↑Muscle [K+]
The effect of AMPD1 genotype on blood flow response to sprint exercise.	Norman et al.	2008	7 males & females	1x30s all out sprint	Suggesting a better circulatory adaptation to exercise in individuals with diminished mAMPD activity, probably due to an AMPD1 genotype-dependent increase in adenosine formation
Effect of high intensity training on exercise-induced gene expression specific to ion homeostasis and metabolism.	Nordsborg et al.	2003	6 males	5.5wk, 23.3 sessions. 15x1 leg kicking for 1min w/3min rec.	Cellular adaptation to high-intensity exercise training may, in part, be induced by transcriptional regulation. After training, the transcriptional response to an exercise bout at a given workload is diminished.
Muscle deoxygenation in aerobic and anaerobic exercise.	Nioka et al.	1998	14 males	1x30s Wingate and a VO_2max test	The results indicate that oxygen was used from the beginning of sprint test, suggesting that the mitochondrial ATP synthesis was triggered after a surprisingly brief duration. One explanation is that prior warm-up was enough to provide the mitochondrial substrates; ADP and Pi to activate oxidative

					phosphorylation by the type IIa and type I myocytes. In addition, transmural pressure created by the muscle contraction reduces blood flow, causing relative hypoxia.
Enhanced sarcoplasmic reticulum Ca^{2+} release following intermittent sprint training.	Ortenblad et al.	2000	9 males (6 males)	20 x 10s all out sprint 50s rec. 3/w for 5 wk	• ↑ peak SR Ca 2+ release, due to an enhanced total volume of SR • SR Ca2+ sequestraction function is not altered.
Combining explosive and high-resistance training improves performance in competitive cyclists.	Paton & Hopkins	2005	9 males (9 males)	5x30s all out sprints and 3x20 explosive step ups. 2-3 x per week. 2min rec	The addition of explosive training and high resistance interval training to the programs of already well-trained cyclists produced major gains in sprint and endurance performance, partly through improvements in exercise efficiency and anaerobic threshold.
The distribution of rest periods affects performance and adaptations of energy metabolism induced by high-intensity training in human muscle.	Parra et al.	2000	10 males	2-7 reps of 15s 45s rec & 30s 12 min rec. all out sprints, 14 training days in 2 weeks. 14 training sessions over 6 weeks w/2days rec in between sessions	High intensity cycling training in 14 sessions improves enzyme activities of anaerobic and aerobic metabolism. Performance did not improve in a short training program that did not include days for recovery, which suggests that muscle fibre suffers fatigue or injury.
Sprint interval and traditional endurance training induces similar improvements in peripheral arterial stiffness and flow-mediated dilation in healthy humans.	Rakobowchuk et al.	2008	20 males & females	4-6 x 30s all out sprints w/4.5min rec. 3/days/wk. 6 wks. 40-60min 65%. 5days/wk	• Improvements in peripheral vascular structure and function comparable to ET. • alterations in central artery distensibility may require a longer training stimuli and/or greater initial vascular stiffness
Effect of acute sprint interval exercise on central and peripheral artery distensibility in young healthy males.	Rakobowchuk et al.	2009	9 males	1x Wingate 4x Wingate w/4.5min rec.	• ↑central artery stiffness • ↓ Peripheral stiffness in exercised limbs well into recovery.
Adrenal medulla responsiveness to the sympathetic nervous activity in sprinters and untrained subjects during a supramaximal exercise.	Zouhal et al.	1998	13 males - 7 athletes and 6 untrained	1x30s all out sprint	Sprinters compared to untrained subjects exhibit a higher responsiveness of the adrenal medulla after a supramaximal test, as shown by higher Amax values and Amax/NAmax ratio. This result suggests that training might increase the adrenal medullary secretory capacity.
A short training program for the rapid improvement of both aerobic and anaerobic metabolism.	Rodas et al.	2000	5 males	2-7 reps of 15s 45s rec & 30s 12 min rec. all out sprints, 14 days in 2weeks	This new protocol, which utilises short durations, high loads and long recovery periods, seems to be an effective program for improving the enzymatic activities of the energetic pathways in a short period of time.

Effect of 6 weeks of sprint training on growth hormone response to sprinting.	Stokes et al.	2004	8 males (8 males)	8 – 10 x 30s sprints 60 – 120s rec	6 weeks of combined speed- and speed-endurance training blunted the human growth hormone response to sprint exercise, despite an improvement in sprint performance.
The growth hormone response to repeated bouts of sprint exercise with and without suppression of lipolysis in men.	Stokes et al.	2008	9 males (9 males)	2x30s all out sprint w/240min rec	Suppressing lipolysis resulted in • ↑GH response to the second of two sprints • Suggesting a potential role for serum FFA in negative feedback control of the GH response to repeated exercise.
Contribution of energy systems during a Wingate power test.	Smith & Hill	1991	6 males	Total of 23 Wingate tests w\7days between each subjects tests	During the entire 30s Wingate, 16% was aerobic contribution, 56% was glycolytic contribution and 28% was ATP-PC contribution. Glycolytic power peaks within the first 15s of high power exercise; also, aerobic metabolism responds quickly during anaerobic exercise and makes a significant contribution.
Unchanged Anaerobic and aerobic performance after short term intermittent hypoxia.	Tabibi et al.	2007	20 males	1x30s Wingate. 15days hypoxic treatment	1h of intermittent hypoxic exposure for 15 consecutive days has no effect on aerobic or anaerobic performance
Metabolic and performance adaptations to interval training in endurance-trained cyclists.	Westgarth-Taylor et al.	1997	8 males	6-9 reps, 12 sets over 6-7 wks, 1min rec. 5mincycling at 80% of VO_2max	Improvements in performance after HIT may occur through an additional recruitment of the motor units needed to sustain exercise at or close to race pace.
Time course of hemorheological alterations after heavy anaerobic exercise in untrained human subjects.	Yalcin et al.	2003	10 males	1x30s Wingate	A single bout of heavy anaerobic exercise may induce significant hemorheological deterioration lasting for up to 12h and thus suggests the need to consider such effects in individuals with impaired cardiovascular function.

Appendix B

Gene expression in muscle tissue

Author	Mode of Exercise	Gene	change	
Bickel 2005	Neuromuscular electrical stimulation	IGF-I IGFBP-4 MyoD D1 P21	↓ ↑ ↑ ↑ ↑	
Eivers et al 2010	Incremental step test		Group A untrained	Group B trained
		LONP1	↑	↓
		PDK1	↑	↓
		PDK2	↓	↓
		PDK4	↑	↑
		SLC2A3	↑	↑
		SLC2A4	↑	↑
		ALDOA	↑	↑
		ENO3	↑	↑
		GAPDH	↑	↑
		LDHA	↑	↓
		PGK1	↑	↑
		HIF1A	↑	↓
		PPARGC1A	↑	↑
		PRKAA2	↑	↓
		COX411	↑	↑
		COX412	↓	↓
		CKM	↑	↑
		AK1	↑	↑
Gibala et al 2009	4 x 30s sprints with 4 min recovery between bouts	PGC-1 alpha	↑	
Louis et al 2007	30 min treadmill run at 75% of VO_2max	FOXO3A Antrogin-1 MuRF-1 Myostatin TNF-alpha	↑ ↑ ↑ ↓ ↑	
Tunstall 2002	63% of VO2 max 60min/day for 9 consecutive days	FAT/CD36 PGC-1 CPT-I B-HAD $FABP_{PM}$/mASPAT PPARγ SREBP—1c PPAR alpha	No change No change ↑ No change No change No change No change No change	
Pilegaard 2000	1-legged knee extensor protocol. 2 min on 5 consecutive days	UCP3 PDK4 Alpha- BC HKII LPL CPT1 GYS HO-1	↑ No change ↑ ↑ ↑ ↑ ↑ ↑	
Richardson et al 2000	30min of knee extension exercise at 50% max work rate	VGEF/18S bFGF/18S	↑ No change	
Yang et al 2004	30 min treadmill run at 75% of VO_2max	MRF4 Myogenin MyoD HKII PDK4 Myf5 CD36 CPT1 GOI	↑ ↑ ↑ ↑ ↑ No change No change No change No change	

Appendix C

Gene expression in Human White Blood Cells

Author	Subject Demographics	Intervention Group (IG)	Comparison Group (CG)	Outcome	Results	Up-regulated genes	Down-regulated genes
Büttner et al., 2007	N=5 Height: 180cm (SD 4.6) Weight: 71.8kg (SD 4.0) Age: 25.4yr (SD 3.5) Training time wk: 6h (SD 2.6) VO_2max: 60.6 ml.min^{-1}.kg (SD 5.3)	Strenuous treadmill exercise test, intensity corresponding to 80% of VO_2peak, until exhaustion. 1-2 weeks later 2nd exercise test at a intensity of 60% of VO_2peak, exercise time identical to the strenuous test.	No comparison group	2weeks before intervention:VO_2max Intervention: Blood taken: pre, post and 1h after both interventions. Leucocyte counts and lactate concentration recorded RNA isolation and microarray measurements: pre and 1h post samples Quantitative real-time PCR: pre and 1h post samples	Lactate [ ] ET: 1.1(SD 0.2) vs 3.4 mmol/L Lactate [ ] MT: 1.3 (SD 0.3) vs 1.2 (SD 0.2) mmol/L Within the group they found a significant change in: WBC number in the ET group post test. Lymphocyte number ET group post test 7 & in MT group 1h post test. Granulocytes/monocyte number in MT group post test and in ET and MT group 1h post test	HSPH1, MMP9, HSPA1A,FLJ22390, CGI-58, IL1R2, SLC2A3, MME, KCNJ15, FOSL2, CASPR3, REPS2, DNAJA1, FLJ23306, C8FW, IL1RAP,CDC42EP2, TGFA, LOC157542, FLJ10357, DAF, NFE2, TSSC3, RERE, MGC31957, NPD009, RNF24, KIAA0963, SLC18A2	PTGDR, ATP5S, BY55, YES1
Conolly et al., 2004	N=15 Age: 25.2 (SD 0.8) Height: 177.6 (SD 1.6) Weight: 76.9 (SD 2.3) BMI: 24.4 (SD 6) VO_2peak ml.kg.min: 37.4 (SD 0.8)	No control group	30min work rate equal to 80% of VO_2peak	Blood samples drawn 30min before exercise, immediately post and 60min post. Serum lactate, circulating leucocytes, serum cytokines & growth factors.	Within the group they found a significant increase in cerum lactate and circulating leucocytes immediate post exercise. Serum cytocine was significantly increased 60min post.	DUSP1, DUSP2, DNAJB1,HSPA1A &B, RTP801, SPON2, CCL3 & 4, CD69, CST7, CTSW, GNLY,GZMA, GZMB, IL-18RAP, IL-2RB, NCR1, NCR3, PRF1, PTGDR, PTGDS, XCL1, XCL2, AKR1CS, EREG, IGFBP7, NCAM1, PDGFRB, CREM, RGS1, TCF8, CLIC3, GPR56	CD22, CYP1B1, NR4A2, GPR86
Radom-Aizik et al., 2008	N=12 Age: 23.3 ±1 Height: 178.2 ±3.2 Weight: 76.5 ±3.9 BMI:24±0.8 VO_2 ml.kg,min: 39.1 ±1.6	30min constant work rate, cycle ergometer, work rate up to 80% of VO_2 peak	No control group	Blood drawn 30min pre training & immediately post exercise. WBC, lymphocytes, monocytes, neutrophils. Neutrophil gene expression	Within the group they found a significant increase in WBC, lymphocytes, monocytes, neutrophils.	RHOB, ANXA1, FEM1B, PDE4B, GZMA, GZMH, PRF1, GZMB,	HSPA1B
Nieman et al., 2007	N=20 Trained cyclists Age: 26 (SD 1.8)	N= 20 Trained cyclists Age: 26 (SD 1.8)	3 consecutive 3h cycling at 57% of VO_2max. Blood sample	Hemoglobin and hematocrit, plasma quercetin, NF-Kb muscle analysis, leucocyte mRNA	Within the group they found no cange in plasma volume, a significant increase in plasma quercetin levels,	IL-6, IL-8, IL1beta, TNF-alpha	

	Weight: 74.7 (SD 0.2) VO_2max ml.kg.min:53.2 (SD 1.2)	Weight: 74.7 (SD 0.2) VO_2max ml.kg.min:53.2	collected 30min pre and 15min post. Muscle biopsies was optained before the 1st session and after the 3rd session	extraction and cDNA synthesis, real-time PCR analysis & muscle total RNA isolation and cDNA synthesis.	plasma cytocine levels decreased significantly over the 3 days.		
Ziekr et al., 2005	N=8 Age: 38.9 (SD11.8) BMI: 23.6 (SD1.8) Athlete population	No control group	One half marathon (21.1km)under competition condition, hilly and demanding terrain.	Blood drawn before, 15min after and 24 h after. Measured: leucocytes, granulocytes, lymphocytes & monocytes.	Within the group they found a significant increase in leucocytes, granulocytes, lymphocytes & monocytes immediatley post and 24h post.	CD81, CD244, Integrin alpha, selectin L, Glutathione S-transferase M3, ICAM2, CD1C, G-protein-coupled receptor 1, MAPKAP K2, CD14, CD19,IL-2beta, CD2, il-8alpha, HSPB1, CD3E, Protein C	
Zeibig et al., 2005	N=6 Junior cross-country skiers, Age: 16.6 ±1.8 Weight: 62.7 ±11,	Low intensity training program, 90 – 120min sessions pr/wk, 12h ave pr/wk, 3wk periods of increase in intensity followed by 1 wk 70-80% reduction in training. Training period lasted for 6mnds. 50% running, 25% cycling & 25% skating	No control group	Venous blood and muscle biopsies pre and post 6mnd training intervention. Plasma carnetine & plasma lipids.	Within the group they found significantly increase in total free fatty acid	CPT1B, CRAT,OCTN2, GRP58, CT1B, CPT2	

Appendix D

Typical training session for female soccer players, wk. 1-4.

Monday:

Speed Drills

- High-knees/glut kicker: 2–3 sets for each workout 15–25 yds for each set
- Resisted running: 3–6 reps for each workout 15–30 sec duration each rep
- *Incline running: 3–6 sets, medium speed/high grade 20–30 sec each set
- *Short high speed sprints: 3–6 sets, over speed/level grade 5–10 sec each set

(* = Treadmill)

Agility/Quickness Drills

- Agility ladder: 2–4 drills, 2–4 sets of each drill
- Step hurdles: 1 forward and 1 lateral drill 2–3 sets of each drill
- Sidestrike box: 2–4 drills, 2–3 sets for each drill
- Lateral/side-stepper: 1–2 drills, 2–4 sets each drill
- Dot drills: 1–2 drills, 2–4 sets of each drill

Wednesday:

Functional Training

- High step-up: 2–4 sets/8–15 reps
- Lateral crossover step-up: 2–4 sets/8–15 reps
- Rotational lunge and reach: 2–4 sets/8 reps
- Medicine ball chop: 2–4 sets/8–12 reps
- Overhead throw: 3 sets/8–12 reps

Friday:

Plyometrics

- Skipping/ankle hops: 15 reps/2 sets(forward/backward, side-to-side)
- Side-to-side box shuffle: 10 reps/2 sets
- Lateral box jumps: 6–8 reps/2 sets
- Lateral cone hops: 6–8 reps/2 sets

(Exercise, intensity and volume change every 2 weeks)

Appendix E

High-quality RNA purified from whole blood or isolated blood cells are required for gene expression studies using real-time polymerase chain reaction (PCR) or microarrays. The RNA obtained for these studies must be highly pure, since accuracy and sensitivity are critical for interpretation of results. Extraction of total RNA was done by a multi step-by-step process listed below, using the RNAasy mini kit from Qiagen.

1. **Homogenization for cell suspension**
    a. Place 1 ml aliquots of the cell suspension in sterile RNase free 1.5 ml microcentrifuge tubes
    b. Centrifuge for 1 minute to pellet the cells
    c. Pour off the supernatant
    d. Add 1 ml of TRIzol to the tubes
    e. Lyse cells by repetitive pipetting
    f. Centrifuge homogenate at 12000 x g for 10 minutes at 4 ^{0}C
    g. Transfer the homogenate in a sterile microcentrifuge tube
    h. Repeat steps f and g twice

    Modification for monolayers

    a) Lyse cells directly in a culture dish by adding 1 ml of TRIzol to every 50-100 mg of sample. Sample volume should not exceed 100 µl.
    b) Pass the cells through a pipette several times
2. **Phase separation**
    a. Incubate samples for 5 minutes at room temperature
    b. Add 0.2 ml of chloroform to each tube
    c. Cap each tube. Shake samples vigorously by hand for 15 seconds
    d. Incubate samples for 5 minutes at room temperature
    e. Centrifuge samples for 15 minutes at 12,000 x g at 4 ^{0}C
3. **RNA precipitation**
    a. Transfer the upper aqueous phase to a fresh tube

b. Add 50 µl isopropyl alcohol, mix, incubate the samples at room temperature for 5 minutes and centrifuge at 12,000 x g for 10 minutes at 4 ^{0}C. Transfer into a new tube. Add 0.5 ml of isopropyl alcohol to precipitate RNA.
c. Incubate for 5-10 minutes at room temperature
d. Centrifuge for 10 minutes at 12,000 x g at 4 ^{0}C. The RNA will form a pellet on the side or bottom of the tube

4. RNA wash

a. Remove supernatant
b. Air dry the pellet with 1 mL 75% ethanol
c. Mix sample by vortexing. The RNA pellet may float
d. Centrifuge at 12,000 x g for 5 minutes at 4 ^{0}C.
e. Repeat steps a, b and c twice

5. Redissolving the RNA

a. Remove supernatant
b. Air dry the pellet for 5-10 minutes. Do not completlely dry out the pellet
c. Dissolve pellet in 30 to 60 µl RNase free water or 0.5% SDS by passing the solution through a pipette tip and incubating for 10 minutes at 55-60 ^{0}C

6. RNA purification

a. Application of DNase directly onto the solid phase medium.
b. Subsequent washing of the affinity medium allows for the removal of salt, protein and other cellular contaminants.
c. Purified RNA molecules become rehydrated following the application of either TE buffer or distilled water to the spin-column and are finally eluted under conditions of low ionic strength

7. RNA Quantification

Appendix F

Day..... Date.....HR.....
Mood state.....
Training
Session no. ..... Start..... Finish.....
Comment.....
Session no..... Start..... Finish.....
Comment.....
Session no..... Start..... Finish.....
Comment.....
Breakfast
Snack
Lunch
Snack
Dinner
Snack

Appendix G

Array location	UQ sample number	My sample code	Group/treatment	Timepoint	RIN	260/280
5445316015_B	2	1245	G1	PRE	7.4	2.03
5445316015_I	13	1545	G1	2	7.5	2.03
5445316015_J	15	1655	G1	3	8.1	1.99
5445316015_K	4	1211	G1	PRE	8.9	1.94
5445316015_H	12	1541	G1	2	8.7	2.14
5445316015_L	14	1651	G1	3	8.9	1.76
6025671001_B	17	2203	G1	PRE	6.6	2.05
6025671001_I	25	2503	G1	2	9.1	2.02
5991413013_E	33	2603	G1	3	6.5	1.8
6025671001_D	19	2206	G1	PRE	6.4	1.75
6025671001_K	27	2506	G1	2	8.3	1.77
5991413013_G	35	2606	G1	3	6.3	1.67
6025671001_F	22	22010	G1	PRE	8.7	1.9
5991413013_B	30	25010	G1	2	8.9	2.09
5991413013_J	38	26010	G1	3	8.2	1.8
6025671001_H	24	32015	G1	PRE	7.5	2.02
5991413013_D	32	25015	G1	2	8.4	2.08
5991413013_K	40	26015	G1	3	7.4	2.01
6025671001_C	18	2204	G2	PRE	7.4	1.95
6025671001_J	26	2504	G2	2	8.3	2.07
5991413013_F	34	2604	G2	3	6.3	1.85
6025671001_E	20	2207	G2	PRE	8.6	1.95
6025671001_L	28	2507	G2	2	NA	1.69
5991413013_H	36	2607	G2	3	NA	1.81
6025671001_G	23	22012	G2	PRE	8.3	1.93
5991413013_C	31	25012	G2	2	9.2	2.04
5991413013_A	29	2508	G2	2	NA	1.94
5991413013_I	37	2608	G2	3	NA	2.04